DEVELOPMENT OF ANTIBODY-LINKED PROBES FOR CHARACTERISATION OF *PSEUDOMONAS* ASSOCIATED WITH SPOILAGE

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Dedicated to Hyacinth Elouise Holness

The great tragedy of science – the slaying of a beautiful hypothesis by an ugly fact.

T.H. Huxley

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ABSTRACT

The growth of micro-organisms in foods is different from that in axenic liquid culture in laboratory media. In natural environments, including food, micro-organisms generally grow in mixed culture and in close proximity to each other, because of which antagonistic or synergistic interactions can occur. To elucidate the behaviour of bacteria within food matrices an understanding of the food structure is required, as foods are complex ecosystems on the micrometer scale. Most processed foods are emulsions and as such are highly structured heterogeneous environments. Antibody-linked probes can be used for the immuno-location of micro-organisms or their products within food matrices to demonstrate the sites at which growth occurs and elucidate the possible bacterial interactions with food components.

The aim of the project was to raise antibodies to spoilage *Pseudomonas* species and to use the developed antibody-linked probes to follow psychrotrophic spoilage *Pseudomonas* within heterogeneous foods. By using antibody-linked probes the natural spoilage of milk and milk products can be followed along traditional lines examining extrinsic parameters but with the additional benefit that the major spoilage organisms can be located within the mixed natural flora. The use of antibodies in this way facilitated the study of a defined natural population and surmounted any adaptive problems associated with introduced organisms. An oil-in-water near-food-grade model was developed to investigate the growth of *Pseudomonas* as it overcame some of the technical problems of using natural cream. *Pseudomonas* species, which grew as colonies within the near-food-grade model, were visualised using fluorescently-labeled antibody-linked probes.

Pseudomonas used to raise the antisera were isolated from psychrotrophically spoiled food and characterised together with isolates retrieved from the environment. The phenotypic characterisation of *Pseudomonas* using classical biochemical tests and API 20NE test strips (BioMerieux) did not produce definitive identifications of the unknown isolates. Nutritional screening of the *Pseudomonas* isolates using commercially produced standardised test microtitration plates (Biolog

MicroPlate™), that contained 95 carbon sources, was carried out. The data produced from the test microtitration plates were analysed using numerical taxonomic methods. The relatedness of the *Pseudomonas* isolates was strongly influenced by the source from which the test isolates originated and did not definitively identify all of the unknown isolates tested. Molecular techniques, ribotyping and amplified ribosomal DNA restriction analysis (ARDRA), based on the genomic fingerprinting of the 16S rRNA gene were evaluated to aid the definitive identification of the *Pseudomonas* isolates but needed a more extensive data base to be useful.

The difficulties encountered in phenotypically identifying food and environmentally isolated *Pseudomonas* species stems from the fact that the *Pseudomonas* genus is now classified according to its ribosomal DNA homology. The classification of the species within the *Pseudomonas* genus is still under review. Robust phenotypic criteria for the identification of all the species within the genus have not to date been defined. In this study, the association of phenotype with environmental source of isolation (whether characterised by nutritional studies or by antibody cross-reaction) demonstrates clearly that more appropriate phenotypic characterisation is required to allow identification schemes to reflect the underlying phylogeny of this group.

Chapter 1

INTRODUCTION

1.1 AIM AND OBJECTIVES

The aim of the present study was to produce antibody-linked probes that could be used to follow a known population of psychrotrophic spoilage *Pseudomonas* species within heterogeneous food systems.

The objectives were to produce antibodies to a known population of *Pseudomonas*. The *Pseudomonas* isolates were to be obtained from food spoiled at refrigeration temperature. Antibody-linked probes were to be developed and applied to real and model food emulsions such that the growth of spoilage *Pseudomonas* could be examined *in situ*. Milk and milk products are natural emulsions that are psychrotrophically spoiled by *Pseudomonas* species. Hence dairy products were chosen as the heterogeneous food systems in which to study psychrotrophic spoilage.

A review of work that has been published on aspects of microbial growth in heterogeneous systems follows. The critique also encompasses food spoilage; conventional microbial detection methods in liquid and structured foods; *in situ* detection methods of microbes within food matrices; antibody-linked probes and an overview of the *Pseudomonas* genus.

1.2 FOOD SPOILAGE

When fruit and vegetables are harvested and animals slaughtered, they begin to decompose. Insects, parasites, chemical contamination, physical damage and/or oxidation may cause decomposition. Decomposition by the action of bacteria, yeasts, moulds and enzymes is highly significant and is brought about by the normal function of micro-organisms attempting to degrade complex organic matter into their constituent components.

Food is considered spoiled when it develops undesirable organo-leptic characteristics such as 'off' flavours and odours, or changes in appearance such as colour, consistency or the production of gas. Food manufacturers not only need to ensure provision of safe food but must also minimise spoilage. Spoiled food, resulting from the failure to control

microbial growth, is costly both economically and possibly in terms of health. To avoid expensive mistakes it is important that food manufacturers can accurately predict the behaviour of micro-organisms in their products. A proper understanding of the behaviour of bacteria within food products requires knowledge of its structure (Heertje, 1993) as foods are complex ecosystems on a micrometer scale.

When applied to food microbiology, ecology can be defined as " *the study of the interactions between the chemical, physical, and structural aspects of the niche and the composition of its specific microbial population*" (Mossel & Struijk, 1992). The food environment is composed of intrinsic factors inherent to the food (e.g. pH, water activity and nutrients) and extrinsic factors external to the food (e.g. temperature, gaseous atmosphere and relative humidity). Intrinsic and extrinsic factors can be manipulated to preserve food and food preservation can be viewed as "the ecology of zero growth" (Boddy & Wimpenny, 1992). The "ecology of zero growth" view does not account for spoilage that occurs without growth, such as spoilage mediated by enzymes.

1.3 BACTERIAL GROWTH IN STRUCTURED FOOD

Established scientific dogma attempts to separate the factors that influence growth, allowing the influence of a single factor to be studied in isolation whilst others remain constant. To achieve this aim requires homogeneous liquid systems which when stirred dissipate concentration gradients and give uniform conditions throughout the test system. Thus liquid axenic cultures are used when forming mathematical models to predict microbial behaviour and growth in foods.

Predictive models assume that bacterial growth rate and survival can be determined by a small number of major intrinsic and extrinsic factors (McMeekin *et al.*, 1993). The effect of these major factors (temperature, preservative concentration, pH and sodium chloride concentration) on bacterial growth is measured and the data are incorporated into mathematical models. The models are then used to predict bacterial growth, lag time and death in specific foods. Extrapolating the results of broth-based experiments to predict the behaviour of micro-organisms in solids may be problematic because it assumes that a liquid model can mimic the conditions that exist within a solid. This, however, is not the case. Mathematical models are invariably fail-safe, as growth in solid foods is slower than predictions (Robins and Wilson, 1994). The failure arises because

the impact of an organism being confined in a fixed position in a solid is not accounted for. Whilst from a food safety point of view this cannot be faulted, it does mean that the models do not accurately reflect growth in solid systems.

Within a solid, diffusion is the dominant form of bulk molecular movement (Wimpenny, 1981). Movement by diffusion is slower than by convection, so the rate of growth in a solid will tend to be slower than that seen in liquid. Fixed positions will create steep diffusion gradients and lead to the occurrence of distance-related effects that would not be present in a liquid system. The growth of spatially confined bacteria will be influenced by the rate at which beneficial substances (nutrients) can diffuse to an individual cell and harmful substances (waste products or inhibitory agents) diffuse away (Wimpenny, 1981). Diffusion will only occur in a heterogeneous system, since the establishment of concentration gradients is a pre-requisite to its function. Heterogeneity within a solid is a multi-factorial concept that can be influenced by the distribution of intrinsic factors, variability of matrix composition, influence of extrinsic parameters and the distribution of other microflora.

Most processed foods are heterogeneous mixtures of raw materials each having different microbiological loading and flora diversity. This heterogeneity of foods means that a number of factors and food components will be unevenly distributed throughout, influencing microbial behaviour (Stringer *et al.*, 1995a). At different sites within food there are differences in pH, oxygen and nutrient levels, water activity and possibly preservatives. The overlapping of numerous diffusion gradients results in the formation of interconnected microenvironments (Dodd & Waites, 1991). Many of the microenvironments may not support microbial growth throughout the bulk of the solid but growth may be possible in isolated pockets. Thus a bacterium located at point A within a food may grow and proliferate. However, another bacterium within the same food at point B may not grow or may die, because of the variation in physical conditions and chemical components within the microstructure. This is illustrated by the food poisoning outbreak in "aerobic" food products (coleslaw, potatoes and sautéed onions) caused by the obligate anaerobe *Clostridium botulinum* (Lund, 1992). Throughout the cooking of these foods oxygen is driven out; on cooling oxygen diffuses back in more slowly, so that regions of the food remain anaerobic. Thus the obligate anaerobe has niches in which it can grow in an apparently hostile environment.

The form that microbial growth takes when food products are colonised depends on the growth form of the micro-organisms concerned and on the structure of the food. In non-liquid food systems position is fixed and the behaviour of the micro-organisms is unlike that of free-living axenic cultures. For example, surface attached micro-organisms, especially those established as biofilms, are 10 - 1000 times more resistant to biocides than free living micro-organisms (Holah *et al.*, 1990; Dhir & Dodd, 1995). Biofilm micro-organisms may be resistant to heat (Frank & Koffi 1990; Dhir & Dodd, 1995), bacteriophages (Hicks & Rowbury, 1987) and antibiotics (Anwar *et al.*, 1989) and can grow at lower nutrient levels (Blenkinsopp & Costerton, 1991). On surfaces a single bacterial cell will develop into a colony and represents a dense population. Heterogeneity within bacterial colonies has been shown to originate from spatially dependant differences in *Salmonella typhimurium* grown within gelatin gel cassettes (Robins *et al.*, 1994). Hence, bacterial populations in solids are more likely to exhibit a wider range of phenotypes than populations grown in liquid culture.

1.4 CONVENTIONAL DETECTION OF MICROBES IN SOLID FOODS

Conventional microbial detection procedures can indicate presence, absence and numbers of micro-organisms but cannot indicate where the micro-organisms are located. The first step in most analyses of solid material is homogenisation by maceration or stomaching. Homogenisation will remove bacteria and matrix components from their original tissue location and all spatial information will be lost as the structural integrity of the food is destroyed. Dilutions of homogeneous suspension are spread onto selective or non-selective plating agar and counts of colony forming units made. Plate counts assumes that the number of visible colonies will be the same as the number of viable cells in the original sample. As some cells may be in clumps or chains (Dodd & Waites, 1992), cell separation may not occur and a single colony on an agar medium may result from more than one cell. Viable counts with standard plate techniques are accepted generally as an accurate reflection of the numbers of micro-organisms within a food, but this is because there is no better or more accurate method available. When resuscitation and/or selective enrichment for a specific micro-organism are used then the results can be even further removed from reality.

Most dairy products, meat pastes, gels and multi-component convenience foods have highly complex physical structures. The majority of processed foods are emulsions (Robins and Wilson, 1994). As already discussed, studies of the growth of micro-organisms in food have been implemented traditionally using homogeneous aqueous systems, disregarding any contributions that the food structure may impart. The development of a model system that incorporates the structure of commonly found foods could be the basis of a more accurate protocol for predicting microbial growth in foods.

1.5 METHODS FOR *IN SITU* DETECTION

Locational studies could identify growth-supporting microenvironments within foods. Such studies would lead to a better understanding of the interaction of micro-organisms with food components and the synergistic or antagonistic relationship between individual micro-organisms (Robins *et al.*, 1994). Once growth-supporting micro-environments have been identified, improved methods to inhibit microbial growth could be developed such as the targeting of preservatives or reduction of the phase(s) supporting growth, thus extending shelf life. *In situ* detection methods can facilitate the direct examination of bacteria within foods and are often more rapid than destructive techniques. They can also elucidate the interaction of bacteria with other cells or food components, establish entry routes of bacteria into the food chain and elucidate the effectiveness of food preservation methods. They can also be used to detect and study those organisms that are difficult to culture within the laboratory.

An assessment of cell viability can be made within food matrices with the use of fluorescently labelled viability stains such as LIVE/DEAD BacLight™ Bacterial viability kits (Molecular Probes, Inc.). The kits use a mixture of nucleic acid stains that differ both in their spectral characteristics and in their ability to penetrate healthy bacterial cell membranes. Organisms with intact cell membranes stain fluorescent green and those with damaged membranes stain fluorescent red.

Biosensors have been developed that detect compounds, such as progesterone, or food-borne pathogens *in situ* within free flowing foods. A disposable amperometric biosensor, based on a screen-printed carbon electrode coated with antibodies was made to estimate the amount of progesterone in cow's milk. The immuno-sensor assayed for

progesterone using progesterone labelled with alkaline phosphatase as a competing marker. The product of the substrate and the alkaline phosphatase reaction was measured electrochemically. The antibody-antigen-binding event directly led to an electrical output. The size of the electrical signal was proportional to the amount of progesterone present in the sample (Pemberton *et al.*, 1997).

Light microscopy has been used in food microbiology for the rapid *in situ* examination of contaminated fruit juice and milk for a number of years. When applied to solid material, sections must first be taken in order to visualise matter from an opaque object. Good sections are obtained from material held rigidly while cut. Material that possesses natural rigidity, for example dry plant tissue, can be sectioned directly. Samples without innate structural rigidity must be manipulated in such a way that they are firm enough to endure sectioning. There are two methods used for sectioning: embedding and cryosectioning. Embedding in paraffin wax or resins involves using fixative agents, such as glutaraldehyde or acetaldehyde, to preserve the structure of the sample. Solvents are then applied to dehydrate the sample that is then treated with hot embedding liquid, which solidifies on cooling. Embedding is a harsh process: the reagents and temperatures used cause structural disruption and displacement of bacteria and food components within the sample which, limits its value for locational studies. Cryosectioning (Dodd & Waites, 1991; Stringer *et al.*, 1995a) is a faster method. In cryosectioning the tissue is fixed by rapid freezing in liquid nitrogen and then kept rigid by maintaining at -20 °C to -40°C whilst being sectioned in a microtome. With cryosectioning, disruption of the food structure and the displacement of food components and micro-organisms is minimised.

Visualisation of food components and naturally occurring micro-organisms within a solid food section is achieved by staining. To identify specific components within sections differential stains are used such as toluidine blue (Flint & Firth, 1988; Dodd & Waites, 1991) or haematoxylin-eosin (Roncales *et al.*, 1991). Although these stains are differential for the food components they are not differential for micro-organisms. Groups of micro-organisms can only be distinguished when there are large differences in morphology. Yeasts and moulds can, therefore, be distinguished from bacteria and bacterial rods from cocci. Differentiation between species can be achieved with the use of specific staining. Gram staining, which is routinely used to classify bacteria into sub-

populations, can be applied to sections (Fernandes *et al.*, 1988; Marcellino & Benson, 1992). A problem with Gram staining is that it requires decolouration with acetone or alcohol, a procedure that will remove lipids and alter the structure within the section.

In situ identification of micro-organisms to the level of genus or even species can be achieved with more specific probes, such as nucleic acid or antibody probes. Nucleic acid probes (Scherthan *et al.*, 1990) bind to complementary sequences of DNA or RNA strands. Probes derived from 16S and 23S rRNA or their genes are commonly used, as regions of the sequence are highly conserved, allowing the differentiation of micro-organisms on a phylogenetic basis (Doyle *et al.*, 1997). The interactions of DNA and RNA probes are highly specific and the probes will bind with high sensitivity to complementary nucleotide sequences. Once a nucleic acid probe has been designed and labelled for a specific bacterial target, the next step would be to fix and permeabilise the cells so that the probe can penetrate the cell wall and locate the target. Permeabilisation of the micro-organisms can involve the use of harsh chemicals, such as formaldehyde. The hybridisation of the probe to the target sequence takes place by incubation for two to three hours. Following hybridisation any non-reacting probe is removed by washing. Long and harsh treatment procedures disrupt food structure and make DNA hybridisation techniques less appropriate for *in situ* detection of bacteria within foods than the use of antibody-linked probes.

1.6 ANTIBODY-LINKED PROBES

Antibodies have been used extensively in microbiology to enumerate and identify bacteria. Antibodies are highly compatible with locational studies as they are specific, versatile and can be used in a spatially resolving manner (Alcock *et al.*, 1992; Dodd & Dainty, 1992; Stringer *et al.*, 1995a). Antibodies can be raised against any structure that can be made immunogenic including proteins, polysaccharides and glycoproteins. Detection and quantification of microbial products and food components can also be achieved.

There is little information available on the application of antibodies to detect and identify bacteria *in situ* within real foods. One reason for limited utilisation has been the rapid development of molecular probes based on complementary DNA sequences targeted against rRNA. Antibody-linked probes have been produced and used to detect

in situ the psychrophilic spoilage organism *Brochothrix thermosphacta* (usually associated with a range of meat products; Stringer *et al.*, 1995a) and nisin (Stringer *et al.*, 1995b), which is a small polypeptide bacteriocin produced by *Lactococcus lactis* usually found in milk.

Antibodies can be labelled, directly or indirectly, with various enzymatic or fluorescent markers that are compatible with differential staining techniques. Fluorescently labelled antibodies allow the visualisation of bacteria at the single-cell level under non-destructive conditions and independently of the growth rate of the cell (Faude and Hofle, 1997).

1.6.1 Structure and function of Antibodies

When challenged by infection or immunisation an animal will defend itself against the foreign invasion. The host defence is initiated by macrophages in the bloodstream. Macrophages are phagocytic and engulf molecules perceived as foreign (antigens). The act of phagocytosis is mediated by T helper cells, which begin a complex chain of responses that result in the stimulation of B-cells. B-cells produce glycoproteins, known as antibodies, which bind to the invading substance or organism. The binding event, between antibody and antigen, labels the foreign invader for destruction via phagocytosis or other host defence systems (complement)(Anon, 2000).

There are five classes of antibodies (or immunoglobulin, Ig) produced by B-cells, IgA, IgD, IgE, IgG and IgM (Table 1.1). Each class of antibody differs in their physiological role and structure (Anon, 2000). The "Y-shaped" structure of an IgG molecule is shown in Figure 1.1.

The immune system encounters numerous and differing substances that are foreign to the host. To label the large number of unknown molecules, antibodies must be extremely diverse. Such versatility emanates from the B-cells that can produce between 10^8 to 10^{10} IgG molecules that differ in the composition of their binding sites. Each individual B-cell produces one immunoglobulin of a single specificity. The differences in antigen specificity between one antibody and another are located in the Fab region of the immunoglobulin molecule (Davey, 1992).

Antibodies have the key attribute of specificity. The requirement to distinguish between self and non-self antigens necessitates a highly discriminating method of recognition at the molecular level (Anon, 2000). The immune system can generate an array of antibody-binding pockets that can accommodate the charge, shape and hydrophobicity of almost any given antigen. Antibody binding occurs through non-covalent interactions (e.g. charge-charge, dipole-dipole, H-bonding and Van der Waals) between the antigen and amino acid residues present in the antibody-binding pocket. The high degree of complementarity displayed during antibody-binding also endows antibodies with high affinities for their antigens.

IMMUNOGLOBULIN	MOLECULAR WEIGHT
IgG1	146 000
IgG2	146 000
IgG3	170 000
IgG4	146 000
IgM	970 000
IgA1	160 000
IgA2	160 000
IgD	184 000
IgE	188 000

Table 1.1 Molecular weight of each class of antibody molecules

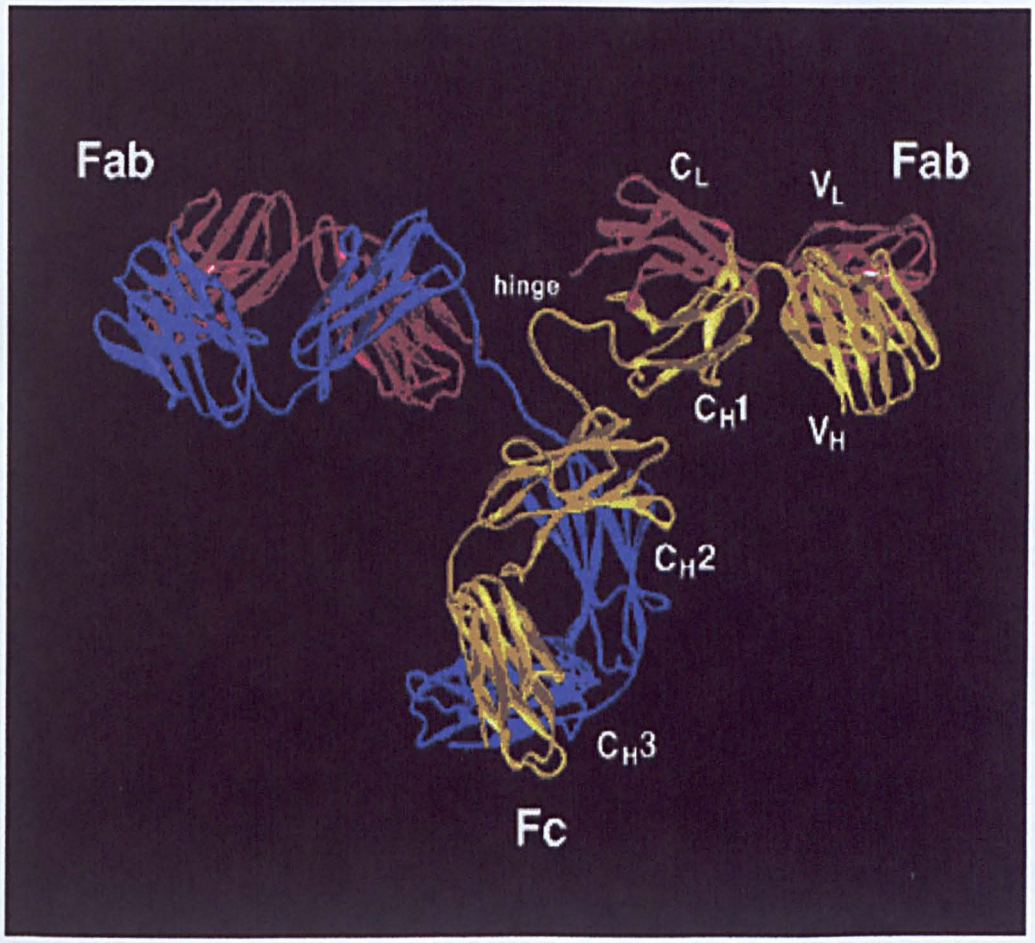

Figure 1.1 A labelled ribbon drawing of the first intact antibody (IgG2A) ever crystallised. IgG antibodies are Y-shaped proteins composed of two heavy chains and two light chains that are joined by disulphide linkages. The IgG molecule can be broken down into two regions, the Fc and Fab. The Fc region, is so called because it is the fragment of the IgG molecule that most readily crystallises, it is involved in effecting the physiological roles the antibody plays. Two identical Fab fragment are present at the ends of the "Y" in every IgG structure. The Fab region contains the antibody binding site and a region of highly conserved amino acids as well as a region of highly variable amino acids. C means constant, V means variable, L means light and H means heavy.

(Picture taken from Anon, 2000)

1.6.2 ANTIBODY PRODUCTION

Antibodies to specific antigens can be generated by either a polyclonal or monoclonal approach. Each approach has advantages and limitations. The choice of method of antibody production should take into account the advantages, limitations and how the antibodies are going to be used. The mechanism of the immune response leads to the stimulation of many different B-lymphocytes resulting in the production of many different antibodies. The mixed population of antibodies produced is known as a polyclonal population. Antibodies originating from a single B lymphocyte are designated monoclonal antibodies.

Polyclonal antisera contain an assortment of antibodies, each having different cellular origin and hence, different specificity. Polyclonal antisera are obtained from an animal (rabbits, goats, guinea pigs or mice), that has been immunised with a specific antigen or immunogen. Up to 13 days after a booster immunisation a sample of blood is removed. The blood cells are removed from the sample by coagulation and the immune response of the resulting antiserum measured. Additional booster inoculations can be made to increase the antibody titre. A disadvantage to this method of antibody production is the variability found in the animal's immune response. All of the antiserum produced from animals immunised with the same immunogen must be extensively characterised.

Monoclonal antibodies overcame the unpredictability of the antibody response by immortalising one B-cell, which would yield one type of antibody only (Kohler & Milstein, 1975). An animal (usually mice) could be immunised with an antigen and once the immune response had been elicited, B-lymphocytes from the spleen could be isolated and fused with an immortal myeloma (tumour-producing) cell. Each of the resultant hybridoma cells would secrete a single antibody. Once a single hybridoma line was selected, it can be propagated in two ways. Firstly, the hybridoma can be injected into a healthy mouse. The hybridoma cells, like the myeloma cells, have the ability to produce tumours and consequently on injection into an animal a tumour would be formed. When the tumour grows it produces ascites, a fluid that is rich in monoclonal antibodies. The ascites method is now used infrequently. Secondly, a more common approach is to allow the hybridoma cells to grow in culture *in vitro*, harvesting the

antibody-rich culture supernatants. The resulting hybridoma population, comprising of clones of the original cell line, should continue to express antibodies indefinitely.

Monoclonal antibodies have a constant affinity for the antigen. Depending on the experimental situation, either a polyclonal or monoclonal antibody approach may be warranted. Polyclonal antibodies are less expensive to produce than monoclonal antibodies. Large amounts of polyclonal antibodies can be produced from the serum of an immunised animal (~10mg/ml) for a finite period (life span of the animal). High affinity polyclonal antibodies can be isolated in a short period of time, two to three months after the initial immunisation. In addition, polyclonal antibodies contain the entire antigen-specific antibody population and in some applications this could be advantageous and in others a decided disadvantage (Anon, 2000). Monoclonal antibodies have certain advantages over polyclonal antibodies, the main one being that the immortal hybridoma cells can be frozen, thawed and re-cultured *in vitro*. Therefore, for a particular monoclonal cell line, there exists a theoretically constant and renewable source of antibodies.

Although monoclonal antibodies have been used to overcome the obstacle of antibody supply, little can be done to control the specificity and affinity of the produced antibody. Methods are being developed which employ molecular biology techniques to facilitate the manipulation of antibody binding sites (Winter & Milstein, 1991). The techniques involve the isolation, amplification and expression of the genes responsible for the variable region of the antibody. The Fab region as a whole or fragments of it (Figure 1.1) are cloned into a bacteriophage, such as M13, which is then allowed to infect *E.coli*. On infection the phage expresses the protein which has full antibody function and can be used in immunoassays (Garrett *et al.*, 1997; Chambers *et al.*, 1999). These functional antibody fragments are known as recombinant antibodies. The potential of this technology is that the antibody-binding properties of the recombinant antibodies can be designed to order. Presently the technology is complex and by no means routine to conduct.

1.6.3 PRINCIPLES OF IMMUNOFLUORESENCE

Coons pioneered the use of fluorescent derivatives of antibodies to trace antigens (Coons *et al.*, 1941, 1942; Coons and Kaplan, 1950; Goding, 1983). Coons and his co-

workers demonstrated that antibodies could be coupled with β anthracene or fluorescein isocyanate and retain their specific antigen-binding properties. The fluorescent antibodies developed could then be used as very sensitive probes to detect and localise antigens (Goding, 1983).

Fluorescence results from a three stage process that occurs in certain molecules variously known as fluorophores, fluorochromes or fluorescent dyes. A fluorescent probe is a fluorochrome designed to localise within a specific region of a biological specimen or to respond to a specific stimulus. The application of fluorescently labelled antibody probes is known as immunofluoresence.

Stage one of fluorescence is excitation. A photon is supplied by an external source, such as a laser or incandescent lamp, and is absorbed by the fluorochrome and transferred to its electrons, which assume a higher energy level. The process distinguishes fluorescence from chemiluminescence, in which energy for the excited state is provided by a chemical reaction. The excited state (stage two) exists for a finite period (Anon, 1998). During this time the fluorochrome undergoes conformational changes and the absorbed energy is released as a photon of lower energy than the initial photon. The emission is known as fluorescence (stage three). Due to energy dissipation during the excited state lifetime, the energy of the emitted photon is lower and therefore of a longer wavelength than the initial photon. The difference in energy or wavelength is known as the Stoke's shift. The fluorescence process is cyclical unless the fluorochrome is irreversibly destroyed in the excited state (photobleaching). The wavelengths that are capable of causing a molecule to fluoresce are known as the excitation spectrum and the wavelengths of emitted fluorescent light, the emission spectrum (Goding, 1983). The fluorescence emission spectrum is independent of the excitation wavelength, due to the partial dissipation of excitation energy throughout the duration of the excited state, although the two spectra may overlap. To isolate emission photons from the excitation photons, wavelength filters are used such that a detector (e.g. photographic film) can register the emission photons and produce a recordable output.

Fluorescence detection can be severely compromised by background signals that originate from sample components (autofluorescence) or from unbound or non-

specifically bound probes (reagent background). Detection of autofluorescence can be minimised by selecting filters with a narrow bandwidth that will increase resolution of the emitted light or by selecting probes that are excited at wavelengths greater than 500nm. At longer wavelengths light scattering by dense media is reduced leading to greater penetration of the excitation light (Anon, 1998)

The introduction of two or more probes to monitor simultaneously different biochemical functions or bacterial cells can be achieved. Multicolour labelling has been applied widely in flow cytometry, DNA sequencing, fluorescence *in situ* hybridisation and fluorescence microscopy. Signal isolation is facilitated by maximising spectral separation of the multiple emissions with the use of appropriate filters. Those fluorochromes with narrow spectral band widths, strong absorption at a coincident excitation wavelength and well-separated emission spectra are ideal for multicolour labelling (Anon, 1998). However, it is not always easy to find dyes with the requisite combination of properties that are both affordable and compatible with existing filters and equipment.

Fluorescent signals can be amplified by increasing the number of fluorochromes available for detection. Enhancement of signals is achieved using avidin-biotin or enzyme-labelled secondary detection reagent in conjunction with fluorogenic substrates. Simply increasing the probe concentration can be counterproductive as it can lead to changes in the optical characteristics of the probe or the reduction of antibody specificity.

1.7 *PSEUDOMONAS*

Pseudomonad is the term given to a non-sporulating, aerobic, rod shaped, Gram-negative, polarly flagellated bacterium. The term is derived from *Pseudomonas*, which is the most important genus of bacteria possessing the aforementioned characteristics (Palleroni, 1986).

The genus *Pseudomonas* includes species with functions of ecological, economic and health related importance. Some species are pathogenic for plants (De Vos *et al.*, 1985; Stead, 1992) while others are opportunistic pathogens of animals or humans (Gilligan, 1991; Palleroni, 1992). A few species exhibit plant growth-promoting and pathogen-

suppressing function and may be exploited for use in biological control (O'Sullivan & O'Gara, 1992). A prominent feature of some species is their metabolic versatility, making pseudomonads attractive candidates for use in bioremediation (Sayler *et al.*, 1990). Numerous studies have described the potential of *Pseudomonas* species to degrade a wide range of compounds (Guerin & Boyd, 1995; Grimberg *et al.*, 1996; Johnsen *et al.*, 1996).

Pseudomonas includes saprophytic and phytopathogenic fluorescent pseudomonads (*Ps. syringae* pathovars and *Ps. viridiflava*), non-pigmented denitrifying species (stutzeri group) and a non-pigmented group (alcaligenes group; Palleroni, 1984). The fluorescent saprophytes include *Ps. putida*, *Ps. aeruginosa* and *Ps. fluorescens* (*Ps. chlororaphis*, and *Ps. aureofaciens* have been classified as being biovars D and E respectively of *Ps. fluorescens*). With the exception of *Ps. aeruginosa* the fluorescent saprophytes are highly heterogeneous (Stanier *et al.*, 1966; Palleroni *et al.*, 1972). *Ps. fluorescens* is thought to have five distinct biovars and *Ps. putida*, two (Stanier *et al.*, 1966). Some strains found in nature fall into well defined categories but cannot be reasonably assigned to any species. Palleroni (1984) stated that the fluorescent saprophytes are the most complex of all groups of aerobic pseudomonads and that a satisfactory taxonomic solution for the internal sub-division into species has not been achieved.

Pseudomonas can produce a range of soluble pigments, notably pyoverdin (fluorescent) and pyocyanin, which is a blue phenazine pigment characteristically produced by *Ps. aeruginosa*. The production of pigments in many instances has taxonomic significance. However, the ability of isolates to produce pigments can be lost or become erratic on repeated sub-cultivation in the laboratory (Palleroni, 1984).

Ps. aeruginosa is the type species of the genus and can be isolated from soil, water or the clinical environment. It is a major cause of hospital acquired (nosocomial) infections. The targets of *Ps.aeruginosa* are immuno-compromised individuals, burn victims, and individuals on respirators or with in-dwelling catheters. Additionally, these opportunistic pathogens colonise the lungs of cystic fibrosis patients, increasing the mortality rate of individuals with the disease. Infection can occur at many sites and can lead to urinary tract infections or pneumonia. Other *Pseudomonas* species isolated from clinical specimens include *Ps. alcaligenes*, *Ps. fluorescens*. *Ps. mendocina*, *Ps. pseudoalcaligenes*, *Ps.*

putida and *Ps. stutzeri* (Howard *et al.*, 1994). *Pseudomonas* species are rarely a cause of infection in healthy individual as the non-invasive nature of *Pseudomonas* species limits pathogenic capabilities.

Pseudomonas includes species of great metabolic diversity and has attracted much attention from a wide range of scientists (Holloway, 1979). Organic chemists were interested in the fact that *Ps.aeruginosa* produces a huge array of degradative enzymes (the genetic information for which is carried on the *Tol* plasmid). The degradative enzymes bio-transform aromatic compounds to less toxic forms and allow further breakdown by other enzymes. The use of such enzymes as anti-fouling agents has obvious benefits. Biochemists and bacterial physiologists have been interested in the intrinsic antibiotic resistance of *Ps. aeruginosa*, which was found to be related to the synergy between low outer membrane permeability and a range of specific drug efflux pumps. *Ps. aeruginosa* also has an intrinsic capacity to tolerate heavy metals and cleaning agents.

Pseudomonas is responsible for the spoilage of a wide variety of low acid foods such as fish, milk, and salad vegetables at refrigeration temperatures. Spoilage of milk products notably by *Ps. fluorescens* can be problematic as the organism can produce heat stable lipases and proteinases. The cells of the psychrotrophs are killed during the heat treatment of milk but the produced lipases and proteases produced during growth survive and may cause spoilage of the products manufactured from the heat-treated milk (Law *et al.*, 1979). At low temperatures the production of lipases and proteolytic enzymes by a psychrotrophic *Ps. fluorescens* strain was at its highest (Peterson & Gunderson, 1960), whilst conversely the metabolism of carbohydrates is reduced. Psychrotrophic spoilage of meat and poultry occurs due to the accumulation of metabolic by-products from the utilisation of amino acids in carcasses. These by-products eventually become detectable as off-odours and slime.

Pseudomonads encompass a large and heterogeneous group of bacteria. The basic criterion for membership of this group fails to circumscribe a single natural group. The very general nature of the membership requirements has led to the formation of many families and genera throughout the 100-year history of pseudomonads, such genera contain species that are only distantly related to each other (as measured by the dissimilarity in their nucleic acid sequences). *Pseudomonas* is one such case.

1.7.1 HISTORY OF *PSEUDOMONAS*

The term *Pseudomonas* means false unit and as the following history of the genus will attest it is aptly titled. Migula first used the name *Pseudomonas* in 1894 and, apart from general morphology, emphasised the importance of flagellation as a means of differentiation. He described them as being "*cells with polar organs of motility. Formation of spores in some species, but it is rare*" (translation given in Palleroni, 1986). A fuller description was given a year later when Migula nominated *Pseudomonas pyocyanea* (later known as *Ps. aeruginosa*) as the type species. Several years later Orla-Jensen (1909) proposed a different system of classification based on physiological characteristics, however, the recommendations were not popular (Ingram & Shewan, 1960).

For the first issue of the *Bergey's Manual of Determinative Bacteriology* in 1925, Buchanan linked physiological concepts with morphological ones to form the basis of a classification. As discussed by Ingram & Shewan (1960) during the early editions of *Bergey's Manual* members of the Eubacteriales that were heterotrophic had straight cells and did not form spores were called Bacteriaceae. The Gram-negative saprophytes of the family (with the exception of cellulose digesters) were then divided according to colour e.g. *Flavobacterium* - yellow, *Pseudomonas* - green and *Achromobacter* - no pigment. Consequently, the convention for classifying the organisms moved towards pigmentation and not type of flagellation. Each genus then incorporated, throughout several editions of the *Manual*, peritrichous and polar flagellation, non-motile and species of undetermined motility. The concept that *Pseudomonas* and *Achromobacter* groups were taxonomically and ecologically linked became established.

In 1926 den Dooren de Jong used nutritional data as a basis for the classification of species. As part of a doctoral thesis he described methodology for extensive phenotypic characterisation of strains that were descriptive, determinative and had significant taxonomic merit. His work highlighted the formidable nutritional versatility of the pseudomonads. In retrospect the adoption of his ideology at this time would have lead to the establishment of sound taxonomic criteria for the classification of the pseudomonads and saved a generation of time in taxonomic chaos. However, the work was published in Dutch and had limited circulation within the English speaking scientific community and hence remained in obscurity. Those who knew of the work were possibly disinclined to adopt de Jong's methodologies as doing so would involve a

large amount of intensive labour (Palleroni, 1992). Stephenson (1939) acknowledged that aspects of the work showed examples of biochemical ingenuity, which indicates that some of the results and conclusions were well known to a few.

In 1936 Kluyver & van Niel insisted that flagellation was an important criterion for classification and that peritrichously flagellated Bacteriaceae represented an evolutionary line different from that of the polarly flagellated Pseudomonadaceae, both of which originated independently from cocci (Ingram & Shewan, 1960). Kluyver & van Niel also maintained that *Pseudomonas* should be regarded as having a strictly oxidative metabolism. Such was the hypertrophy within the Pseudomonadaceae at that time that they suggested that it should contain 25 genera grouped within three tribes. Some of the criticisms of Kluyver & van Niel to the Bergey's Manual approach became accepted and the text adopted a more phylogenetic approach in the next edition (5th). All polarly flagellated species were removed from the *Achromobacter* genus and grouped within the *Pseudomonas* genus; all peritrichously flagellated species were removed from the *Pseudomonas* genus and housed within *Achromobacter*. The genus *Achromobacter* was placed within the Bacteriaceae family and was separated from the Pseudomonadaceae by the Coccaceae. Hence *Pseudomonas* and *Achromobacter* were divorced from each other (Ingram & Shewan, 1960).

By the 6th edition of Bergey's Manual further changes were made. The genus *Pseudomonas* expanded with the incorporation of nearly 100 plant pathogens and, for the first time, non-pigmented strains (23). Together with the green plant pathogenic strains, the genus *Xanthomonas* containing yellow plant pathogens with polar flagella were included in the Pseudomonadaceae.

Throughout the 1950's advances were made in the field of bacterial biochemistry and several *Pseudomonas* species were studied in depth. Simple tests were developed that facilitated the formation of schemes to differentiate between groups or genera (Hugh & Leifson, 1953). On the basis of these biochemical advances the 7th edition of Bergey's Manual lead to further sub-divisions of the Pseudomonadaceae which related to themes expounded earlier by Kluyver & van Niel.

In the 1953 edition of the Bergey's Manual (7th), the Pseudomonadaceae contained 12 genera (*Pseudomonas, Xanthomonas, Acetobacter, Aeromonas, Photobacterium, Azotomonas, Zygomonas, Protaminobacter, Alginomonas, Mycoplanar, Zoogloea* and *Halobacterium*). The definition of *Pseudomonas* in the 7th edition was, "*cells monotrichous, lophotrichous or non-motile Gram negative, frequently develop fluorescent, diffusible pigments, of a greenish, bluish, violet, lilac, rose, yellow or other color. Sometimes the pigments are bright red or yellow and non-diffusible; there are many species that fail to develop any pigmentation. The majority of species oxidize glucose to 2-ketogluconic acid or other intermediates. Usually inactive in the oxidation of lactose. Nitrates are frequently reduced either to nitrites, ammonia or free nitrogen*".

Alongside the strategies adopted by the Bergey's Manual editors, Prevot (1933) in France proposed an alternate scheme that stressed the importance of morphology and relegated the emphasis of physiology for the differentiation of species (Ingram & Shewan, 1960). In 1948 Prevot stated that it was wrong to group together anaerobic organisms that attacked cellulose whose method of sporulation was different. Brisou (1958), a collaborator of Prevot, later used the theories of Prevot as the basis of a classification of the Pseudomonadaceae. The fact that pigmentation was reinstated as a primary criterion and that the type of flagellation was unimportant (although non-motile species were not included) made his schemes reminiscent of the early editions of the Bergey's Manual (Ingram & Shewan, 1960). The non-pigmented polar species of *Pseudomonas* rejoined the peritrichous species in *Achromobacter* and the non-motile species (many of which were from *Achromobacter*) were gathered in the newly formed genus *Acinetobacter*.

The widely divergent views held on the Pseudomonadaceae are reflected in the various proposed schemes of taxonomy and resolution of this problem was hampered by the lack of readily applicable discriminatory tests. The development of new tests and equipment (e.g. phase contrast and electron microscope) were beneficial. Workers in the field during the 1950's spent little time speciating isolates because as stated by Ingram & Shewan (1960) "*It seems that most of the Pseudomonas and Achromobacter species described in the current lists of Bergey's Manual or of Brisou are illusory*"

The late 1950's saw the resurgence of interest in taxonometric methods used in classification, probably precipitated by the increased availability of electronic digital

computers (Sokal & Sneath, 1963). Numerical taxonomy was defined as "*the numerical evaluation of the affinity or similarity between taxonomic units and the ordering of these units into taxa on the basis of these affinities*" (Sokal & Sneath, 1963). The fundamental concepts behind numerical taxonomy were objectivity (Adansonian principle, meaning the equal weighting of all characters) and repeatability (standardised tests), such that various workers who independently characterised an organism using the same tests would yield the same conclusions. This approach would lead to the formation of stable phenetic groupings of bacterial species. Numerical taxonomic aims were not to produce a phylogenetic grouping but where there was doubt about which of two organisms was most likely to have been the ancestor of a third, numerical taxonomy could give objective information on which to base a decision (Sokal & Sneath, 1963). Sneath (1957a) argued that many schemes of bacterial taxonomy were not classifications but catalogues and stipulated that there was no reason why one feature should be given greater weight in classification than any other. He claimed that it was not necessary to know the evolutionary history of organisms to classify them in a scientific manner. When considering the state of the *Pseudomonas* and *Achromobacter* genera in particular, Ingram & Shewan (1960) suggested that it may be necessary to relinquish the old concept of the genus as a distinct systematic unit and apply pragmatically the Adansonian techniques of Sneath (1957b).

Jessen (1965) reiterated the thoughts of Ingram & Shewan (1960) when he stated that most of the methods used to devise a rational taxonomic system were inappropriate "*as there were good reasons to doubt the homogeneity as well as rank of the genus Pseudomonas, a taxonomic revision is clearly required. This will not only necessitate extensive investigations of the presently accepted constituents of the genus, but also comparative examinations of related genera, using exactly the same methods*". The taxonomic turmoil did not dissuade many workers from describing new species, in spite of the cautionary views of Jessen. The use of insignificant tests as the basis of determinative schemes for the classification of the pseudomonads together with the description of many new species on the basis of meaningless characters did little to enhance the taxonomic status of the group (Palleroni, 1978). Many of the species names that appeared in the 6th and 7th editions of the Bergey's Manual have little or no meaning today as the original examples have been lost. The description of the redundant organisms was so poor that further isolates could not be identified from nature.

The classification of *Pseudomonas* throughout this time had been at best poorly characterised and at worst highly confusing. Even those species that had been well described enjoyed a precarious stability, since from time to time radical revisions in genus definition were implemented by taxonomists (Palleroni, 1992). It was in the 1960's that a move towards clarity within the genus was initiated.

In the early 1960's R. Stanier at the University of California at Berkeley began a collection of 297 pseudomonads biotypes that contained representatives of various physiological groups. All the organisms were nutritionally screened with standardised methods, a process that was strongly influenced by and substantiated the work of den Dooren de Jong (Stanier *et al.*, 1966). The resultant data revealed many previously unrecognised characters of taxonomic significance. The grouping of biotypes into species had been mainly done on the subjective evaluation of the data. Stanier *et al.* (1966) expressed the opinion that nutritional characters, that numerical taxonomists would treat as having equivalent weight, were in fact far from equivalent to one another when the underlying enzymic mechanisms were taken into consideration. The analysis led to an internal sub-division of the genus into species. So, after nearly 70 years the Berkeley group were able to sub-divide rationally the *Pseudomonas* genus into species. It is interesting to note that in later years an extensive numerical treatment of the phenotypic data derived from the Berkeley collection was conducted by Sneath *et al.* (1982), the results of which were in very good agreement with the groupings of Stanier and his co-workers.

Improved accuracy in the taxonomy of pseudomonads continued to occur due to the improvement and wide usage of genetic techniques (Palleroni *et al.*, 1973; Li *et al.*, 1993). Throughout the early 1970's members of the Berkeley group confirmed the scheme of phenotypic classification by DNA hybridisation studies. However, many strains of *Pseudomonas* appeared to share relatively low levels of homology with the other *Pseudomonas* species. Johnson & Palleroni (1989) used various methodologies to confirm the low genetic similarity within the genus. To resolve the heterogeneity amongst the members of the genus the conservative regions of the genome such as rRNA genes, were used in DNA-rRNA hybridisation experiments. The use of rRNA in hybridisation with chromosomal DNA indicated that the *Pseudomonas* genus could be divided into five

phylogenetically distant groups (Palleroni, 1973). Results from the hybridisation experiments showed that organisms from other genera, such as *Escherichia* and *Xanthomonas*, were more closely related to some *Pseudomonas* species than to *bone fide* members of the *Pseudomonas* genus themselves. According to Palleroni *et al.* (1973) each homology group deserved to be categorised as an independent genus and possibly a family or order assignment. Phenotypic characters were found that could differentiate between the *Pseudomonas* species within the group. However, enough useful characters to elucidate all species of any one rRNA group (with the exception of RNA group IV) from those belonging to other groups (Palleroni, 1992) were not found. Work reported by De Vos & De Ley (1983) and De Vos *et al.* (1989) allowed additional *Pseudomonas* species to be assigned to each of the five rRNA groups and inappropriately classified species were excluded.

The work of the Berkeley group and subsequent workers with regard to substantiating a stable phylogenetic and phenotypic classification system for *Pseudomonas* was arguably the most important throughout the history of taxonomy of the genus. At the time however, no major changes were initiated in *Pseudomonas* taxonomy but the approaches that were used to clarify the complexities of the group were applied to other bacterial genera to describe their phylogenetic relationships (Palleroni, 1992).

The 8th edition of the Bergey's Manual, published in 1974, divided the Pseudomonadaceae into four genera, *Pseudomonas*, *Xanthomonas*, *Zooglea* and *Gluconobacter*. The *Pseudomonas* genus was defined as being straight or curved rods, chemo-organotrophs (respiratory metabolism, never fermentative), Gram negative, motile by polar flagella (monotrichous or multitrichous), strict aerobes except those species that can denitrify using nitrate as an alternate electron acceptor. The use of mode of flagellation as means of identifying evolutionary relationships was dismissed within this edition. The authors recognised that some species designated as *Pseudomonas* were less closely related to one another than they were to some peritrichously flagellated species. The number of genera within the Pseudomonadaceae was much reduced from the previous edition with many of the groups placed within other families. The genus *Hydrogenomonas* was no longer recognised as a distinct group and many of the species contained therein were placed into the *Pseudomonas* or *Alcaligenes* groups. The tests listed to produce a determinative key were those of Stanier *et al.* (1966). Passing reference was

made to the attempts to establish significant criteria for defining species by extensive comparative studies. However, it was felt that the work was too recent to be included.

In 1984 the current Bergey's Manual of Systematic Bacteriology was published (volume one of four of a new format). The Pseudomonadaceae was divided once again into four genera, *Pseudomonas, Xanthomonas, Frateuria* and *Zoogloea*. *Gluconobacter*, which was previously placed within this family, was assigned to the Acetobacteraceae and its place taken by a newly created genus *Frateuria*. No natural relationships had been found between *Zoogloea* and the other three genera and inclusion of the genus in the family is only tentative.

Norberto Palleroni authored the Pseudomonadaceae section of the new format Bergey's manual. He admitted that "*from a practical determinative standpoint, a clear-cut circumscription of the family by classical phenotypic criteria has become more difficult than ever before, and differentiation of the genera may require specialised techniques which are still beyond the reach of many laboratories*". The description of the *Pseudomonas* genus was very much as the 8th edition with the one or two inclusions such as that the oxidase reaction could be positive or negative. The taxonomic classification within the genus now followed the ground-breaking proposals made by Palleroni *et al.* (1973), with the *Pseudomonas* species categorised into five rRNA groups. The internal sub-division of the genus, in the main, was in agreement with the phenotypic groupings outlined in the 8th edition. There are still however, a large number of species that are not included in the five rRNA groups as their natural relationships are unknown at present.

1.7.2 CURRENT CLASSIFICATION OF *PSEUDOMONAS*

Kersters *et al.* (1996) published a concise overview of the current knowledge of the phylogenetic relationships, classification and nomenclature of all the validly described species of the *Pseudomonas* genus as defined in the current Bergey's Manual. Lists were produced containing up-to-date genus and species names as of April 1996 (Tables 1.2 & 1.3). Results of polyphasic taxonomic studies (including 16S rRNA sequence analysis, DNA:DNA hybridisation and rRNA:DNA hybridisation) have lead to the majority of *Pseudomonas* species being assigned to various rRNA groups (Palleroni *et al.*, 1973; Palleroni, 1984) and sub-classes of the *Proteobacteria* (Stackebrandt *et al.*, 1988; Figure 1.2). Major changes in the nomenclature of pseudomonads have occurred.

The name *Pseudomonas* is reserved for members of the rRNA group I and they belong to the γ sub-class of the *Proteobacteria*. The main body of the rRNA group II species belong to the β sub-class of the *Proteobacteria* and have been assigned to the newly created genera *Burkholderia* (the type species is *Burkholderia cepacia*) and *Ralstonia* (the type species is *Ralstonia picketti*). Group III organisms, which also belong to the β sub-class, have been reclassified into the Comamonadaceae family. The Comamonadaceae family contains the genera *Acidovorax*, *Comamonas* and *Hydrogenphaga* that were all previously defined as *Pseudomonas* species. The members of the rRNA group IV belong to the α sub-class of the Proteobacteria and have been allocated to the new genus *Brevundimonas*. Finally, rRNA group V species were initially located within the *Xanthomonas* genus and then placed into another new genus *Stenotrophomonas*. The genus *Stenotrophomonas* is located in the γ-β sub-class of the Proteobacteria. Other *Pseudomonas* species have been transferred to newly proposed genera such as *Chryseomonas*, *Oligotropha* and *Zavarzinia*, the phylogenetic relationships of which are not yet known in detail.

The *Pseudomonas* genus is now restricted to 35 or so species that are related, phenotypically and genotypically, to the type species *Pseudomonas aeruginosa*. The species contained within the genus are mainly saprophytic or pathogenic to plants or fungi. The *Pseudomonas sensu stricto* contain both fluorescent and non-fluorescent species. Extensive polyphasic studies indicate that *Ps. fluorescens*, *Ps. marginalis*, *Ps. putida*, *Ps. stutzeri* and *Ps. stanieri* are heterogeneous species (Vancanneyt *et al.*, 1996) and the inter-species relationships and sub-groups are not yet fully elucidated. *Ps. aeruginosa* is a highly homogeneous species. The rRNA:DNA hybridisation studies (DeVos & DeLey, 1983) and 16S rRNA sequence analysis (Moore, 1990) show that the genus *Pseudomonas sensu stricto* is phylogenetically related to the free-living nitrogen-fixing bacteria of the genera *Azotobacter* and *Azomonas*.

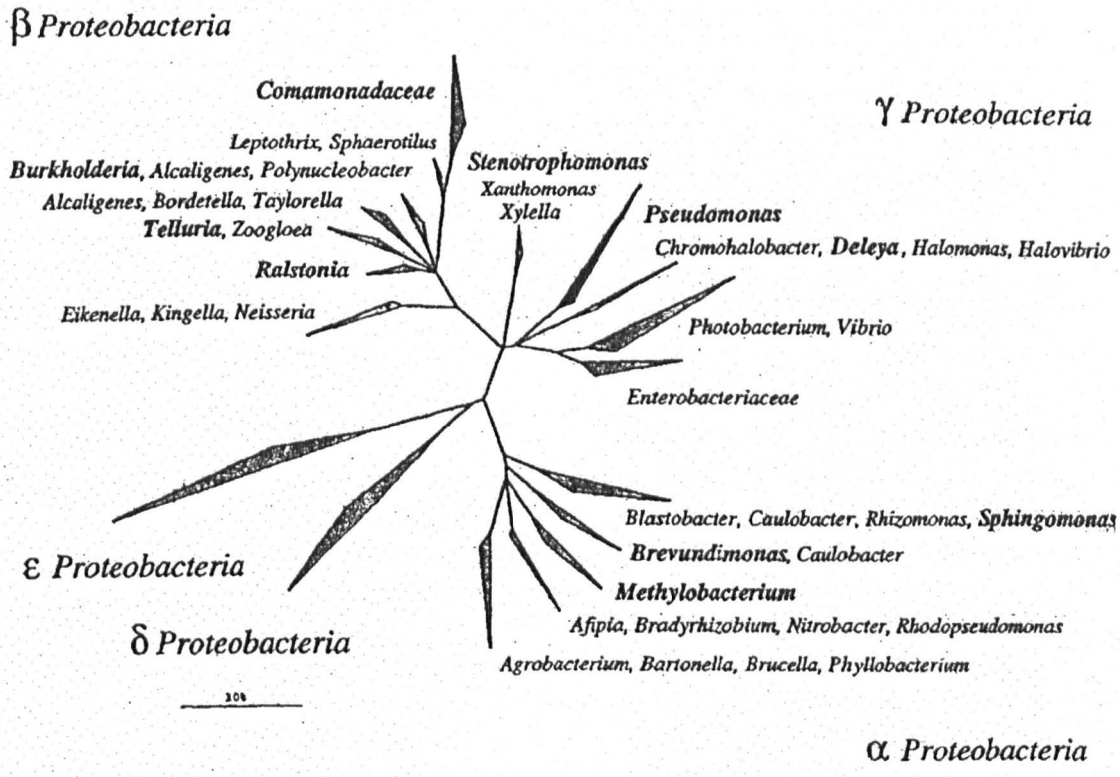

Figure 1.2 Showing the phylogenetic relationships within the Proteobacteria (based on diagram from Kersters *et al.*, 1996)

Table 1.2 Current classification rRNA Group 1 - *Pseudomonas sensu stricto*

(based on Kersters *et al.*, 1996)

Previous Name	Current classification	Previous Name	Current Classification
P. aeruginosa	*P. aeruginosa*	*P. mucidolens*	*P. mucidolens*
P. agarici	*P.agarici*	*P. oleovorans*	*P. oleovorans*
P. alcaligenes	*P. alcaligenes*	*P. perfectomarina*	*P. stutzeri*
P. amydali	*P. amydali*	*P. pseudoalcaligenes*	*P. pseudoalcaligenes*
P. anguilliseptica	*P. anguilliseptica*	*P. putida* (2 biovars)	*P. putida* (2 biovars)
P. asplenii	*P. asplenii*	*P. resinovorans*	*P. resinovorans*
P. aureofaciens	*P. chloroaphis*	*P. savastanoi*	*P. savastanoi*
P. caricapapayae	*P. caricapapayae*	*P. stanieri*	*P. stanieri*
P. chloroaphis	*P .chloroaphis*	*P. stutzeri*	*P. stutzeri*
P. cichorii	*P. cichorii*	*P. stutzeri* genomovar 6	*P. balearica*
P. citronellolis	*P. citronellolis*	*P. synxantha*	*P. synxantha*
P. coronafaciens	*P. coronafaciens*	*P. syringae*	*P. syringae*
P. corrugata	*P. corrugata*	*P.syringae* pv *.avellanae*	*P. avellanae*
P. ficuserectae	*P. ficuserectae*	*P. taetrolens*	*P. taetrolens*
P. flavescens	*P. flavescens*	*P. tolaasii*	*P. tolaasii*
P. fluoresens all biovars	*P. fluoresens* all biovars	*P. viridiflava*	*P. viridiflava*
P. fragi	*P. fragi*	*Pseudomonas luteola*	*Chryseomonas luteola*
P. fuscovaginae	*P. fuscovaginae*	*Pseudomonas oryzihabitans.*	*Flaviomonas oryzihabitans*
P. lundensis	*P. lundensis*	*Pseudomonas marina*	*Deleya marina*
P. marginalis all pathovars	*P. marginalis* all pathovars	*Pseudomonas beijerinckii*	*Deleya-Halomonas* rRNA lineage
P. meliae	*P. meliae*	*Pseudomonas doudoroffi*	Related to Aeromonadaceae
P. mendocina	*P. mendocina*		

Table 1.3 Classification of the rRNA groups II - V pseudomonads
(based on Kersters *et al.*, 1996)

Previous Name	Current classification	rRNA Group
P. andropogonis	*Burkholderia andropognis*	II
P. caryophylli	*Bu. caryphylli*	II
P. cepacia	*Bu. cepacia*	II
P. cocovenenans	*Bu. cocoovenenans*	II
P. gladioli	*Bu. gladioli*	II
P. glumae	*Bu. glumae*	II
P. mallei	*Bu. mallei*	II
P. plantarii	*Bu. plantarii*	II
P. pseudomallei	*Bu. pseudomomallei* *Bu. vandii* *Bu. vietnamiensis*	II
P. woodsii	*Bu. andropogonis*	II
P. picketti	*Ralstonia picketti*	II
P. solanacearum	*R. solanacearum*	II
P. acidovorans	*Comamonas acidovorans*	III
P. testosteroni	*C. testosteroni*	III
P. delafieldii	*Acidovorax delafieldii*	III
P. facilis	*A. facilis*	III

TABLE 1.3 Classification of the rRNA groups II - V pseudomonads cont'd

PREVIOUS NAME	CURRENT CLASSIFICATION	rRNA GROUP
P. flava	*Hydrogenophaga flava*	III
P. palleronii	*H. palleronii*	III
P. pseudoflava	*H. pseudoflava*	III
P. saccharophila	Related to Comamonadaceae	III
P. diminuta	*Brevundimonas diminuta*	IV
P. vesicularis	*Br. vesicularis*	IV
P. maltophila	*Stenotrophomonas maltophila*	V

1.7.3 PSYCHROTROPHIC SPOILAGE OF MILK AND MILK PRODUCTS

Fresh milk from a healthy cow is virtually free from bacteria and will be contaminated as it leaves the udder with bacteria in the teat channel. Micro-organisms capable of spoiling milk are ubiquitous and originate from outside the udder, milking machines, dust particles, the cow's hide, animal bedding and in the soil. The composition of the contaminating flora relates to the cleanliness of the animal's environment and that of the surfaces with which the milk comes into contact. During udder inflammation (mastitis) milk may become unfit for human consumption due to the heavy microbial contamination. Even within the best milk production systems it is impossible to completely exclude bacteria from milk (Cousin, 1982).

Milk is a very good medium for bacterial growth due to the high water content, near neutral pH and variety of available nutrients. The components of milk include lactose, fat, protein, citrate, minerals and various non-protein nitrogenous compounds. Milk leaves the udder at about 37°C and on contamination bacteria may begin to proliferate. Many micro-organisms cannot utilise lactose and therefore rely on proteolysis or lipolysis to obtain carbon and energy.

Raw milk contains microbial inhibitors such as lactoferrin and lactoperoxidase. Lactoferrin is a glycoprotein and acts by chelating iron. Human milk contains over 2 mg/ml of lactoferrin but it is of lesser importance in cow's milk, which contains 20-200 μg/ml and the presence of citrate in cow's milk competes with lactoferrin for binding iron (Doyle *et al.*, 1997).

The most effective natural microbial inhibitor in cow's milk against Gram-negative rods including *Pseudomonas*, is the lactoperoxidase system. Lactoperoxidase is an enzyme that catalyses the oxidation of thiocyanate and the simultaneous reduction of hydrogen peroxide and results in the accumulation of hypothiocyanite. Hypothiocyanite leads to enzyme inactivation and structural damage to the cytoplasmic membrane (Wolfson & Sumner, 1993; Doyle *et al.*, 1997). Both lactoperoxidase and thiocyanate are present in milk during secretion, whereas hydrogen peroxide is formed in milk by lactic acid bacteria. Hydrogen peroxide is the limiting substrate for the reaction and can be added

via seeding the milk with lactic acid bacteria. The antibacterial effect depends on the amount of thiocyanate present and the temperature. When the thiocyanate is depleted the bacteria can start to proliferate within 4 hours at 30°C and 72 hours at 5°C. The antibacterial component, thought to be a derivative of thiocyanate oxidation, is readily destroyed at 60°C and is not present in pasteurised milk (Cousin, 1982).

Rapid chilling to below 4°C inhibits the growth of bacteria in the milk, thereby significantly improving its keeping qualities. The refrigerated storage of fluid milk can be extended over a long period from the time the product is collected from the animal and stored in bulk tanks on the farm until the time it arrives at the processing plant. Storage of raw milk at refrigerated temperatures prior to manufacturing selects for the growth of psychrotrophs (Fairbairn & Law, 1986).

The terminology pertaining to micro-organisms that are able to grow at temperatures close to 0°C have been confused since the start of the 20th Century (Brock & Madigan, 1988). Schmidt & Nielson (1902) termed micro-organisms that can grow at 0°C, psychrophiles (cold loving). The term implies that the micro-organism grows optimally at low temperatures. Subsequent workers objected to the definition of psychrophile as many micro-organisms that can grow at low temperatures grew best at 20°C or higher. Terms such as mesophile (mid-range temperature optima) and theromophile (high-range temperature optima) defined microbial growth at optimum temperatures. Mossel applied the term psychrotroph (cold thriving) to micro-organisms able to grow on solid media at 5°C or below regardless of their optimum temperature (Eddy, 1960). In 1975 Morita suggested that mesophilic micro-organisms that can grow at 0°C be more correctly termed psychrotrophic. The term psychrophilic is now reserved for micro-organisms with an optimum growth temperature of 15°C, a maximum growth temperature of 20°C and a minimum growth temperature of 0°C or below. In the dairy industry a psychrotroph is defined as a micro-organism that is able to grow at 7°C or below, regardless of their optimum growth temperature (Eddy, 1960). The dairy industry definition of psychrotroph will be employed throughout this text.

Psychrotrophs are ubiquitous in nature but are rarely found in the udder of the animal and include bacteria, yeasts and moulds. Psychrotrophic bacteria can be Gram positive,

Gram negative, spore formers aerobic, anaerobic or facultatively anaerobic. Psychrotrophs account for less than 10% of the initial raw milk microflora, however, they grow rapidly and dominate the flora during refrigeration (Shah, 1994). *Pseudomonas* is the major psychrotrophic spoiler of milk and milk products at refrigeration temperatures, in particular *Ps. fluorescens* (Cousin, 1982; Shah, 1994). Garcia *et al.* (1989) followed the incidence of pseudomonads in supplies of raw milk to a single factory for a year and found that *Pseudomonas* accounted for 67% of the total psychrotrophs, the majority belonging to *Ps. fluorescens* (Shah, 1994). Other genera found included *Acinetobacter*, *Aeromonas* and *Micrococcus*.

Cells of psychrotrophic *Pseudomonas* do not survive pasteurisation or ultra high heat treatment. Strains of *Pseudomonas* are problematic due to their ability to produce thermally resistant enzymes, lipases and proteases, which resist heat processing. The activity of the lipases and proteases do not stop after the bacteria have been killed, resulting in bad smells and tastes (Law, 1979; Fairbairn & Law, 1986). The current practice of storing milk at 4-7°C for up to four days prior to processing is sufficient for large microbial populations to arise and produce significant amounts of enzymes. The generation time of psychrotrophic *Pseudomonas* is about 8 hours at refrigeration temperature (Gounot, 1991).

Proteolytic activity in milk usually results in a bitter flavour after digesting casein into soluble peptides. Lipolytic activity can produce a range of flavour defects that can be described as malty, cheesy, fishy or unclean (Shah, 1994). Gelation and bitter flavours of UHT milk are associated with proteolytic enzymes (Fairbairn & Law, 1986). Other effects of enzymic activity include reduced frothing of milk in cappuccino machines, bitty cream, longer churning time for butter and batch failures in yoghurt and cottage cheese production (Cousin, 1982)

The growth and metabolic activity of *Pseudomonas*, originating from post pasteurisation contamination (PPC), is the single most detrimental factor determining the keeping quality of refrigerated pasteurised milk (Shah, 1997). A fruity flavour is associated with PPC with *Ps. fragi*, which produces ethyl esters. PPC occurs as the milk passes from the pasteuriser to the final containers. *Pseudomonas* is well adapted to survival in the milk-processing environment and is able to adhere strongly to the surfaces of the milk

processing equipment. When attached to the surfaces of the processing equipment *Pseudomonas* can resist the action of cleaning fluids. With close adherence to good manufacturing practice a frequency of PPC of 0.001-1 cfu/ml with *Pseudomonas* is common in commercial processing units (Sorhaug & Stepaniak, 1997). Under laboratory pasteurisation conditions PPC can be eliminated to yield milk with a shelf-life of 3-5 weeks at 4 -7°C which is much longer than the typical commercially pasteurised milk with a shelf-life of 8-12 days (Sorhaug & Stepaniak, 1997).

The natural spoilage of dairy products can be monitored with antibody-linked probes along traditional lines, examining extrinsic parameters, with the additional benefit that the major spoilage organisms can be located within the mixed natural flora. The use of antibodies in this way would overcome any adaptive problems associated with introduced organisms in model systems.

MATERIALS AND METHODS

2.1 BACTERIAL MEDIA USED:

2.1.1 BRAIN HEART INFUSION AGAR.

Brain Heart Infusion (BHI) agar was prepared by the addition of bacteriological agar No.1 (7.0 g, Oxoid) to BHI broth (500 ml) and then autoclaved (121°C, 15 min).

2.1.2 BRAIN HEART INFUSION BROTH.

BHI broth (18.5 g, Oxoid) was made up to a final volume of 500 ml with reverse osmosis water and autoclaved (121°C, 15 min).

2.1.3 *PSEUDOMONAS* CFC AGAR

Pseudomonas agar base (24.2 g, Oxoid) was suspended in reverse osmosis water (500ml) followed by the addition of glycerol (5ml). The media was autoclaved (121°C, 15 min) and tempered to 55°C. The contents of 1 vial of *Pseudomonas* CFC supplement was rehydrated with sterile reverse osmosis water (2ml) and added to the tempered agar base which was then mixed well before being poured into sterile petri dishes.

2.1.4 MAXIMUM RECOVERY DILUENT.

Maximum Recovery Diluent (MRD) (4.75 g, Oxoid) was made up to a final volume of 500 ml with reverse osmosis water and autoclaved (121°C, 15 min).

2.1.5 MOTILITY AGAR

Yeast extract (0.1 g /l, Oxoid), calcium chloride (0.9 g/l) and bacteriological agar No. 1(3.0 g/l, Oxoid) was mixed together in reverse osmosis water and autoclaved (121°C, 15 min).

2.1.6 NUTRIENT AGAR.

Nutrient agar (NA) (14 g, Oxoid) was made up to a final volume of 500 ml with reverse osmosis water and autoclaved (121°C, 15 min).

2.1.7 NUTRIENT BROTH.

Nutrient broth (NB) (13 g, Oxoid) was made up to a final volume of 500 ml with reverse osmosis water and autoclaved (121°C, 15 min).

2.1.8 SKIM MILK AGAR

Double strength NA (500ml) was sterilised (121°C, 15 min) and then tempered to 55°C. Skim milk powder (50 g, Marvel) was added to reverse osmosis water (500 ml), sterilised (115°C, 10 min) and then tempered to 50°C. The sterile double strength NA and 10% skim milk solution were gently mixed together (1:1) before being poured into sterile petri dishes. The surfaces of freshly poured plates were flamed with a Bunsen burner to remove large bubbles.

2.1.9 TRYPTONE SOYA AGAR

Tryptone soya agar (TSA)(40g, Oxoid) was suspended in reverse osmosis water (1 litre) and autoclaved (121°C, 15 min).

2.1.10 TRYPTONE SOYA BROTH

Tryptone soya broth (TSB) (30g, Oxoid) was dissolved in reverse osmosis water (1 litre) and autoclaved (121°C, 15 min).

2.2 STORAGE OF *PSEUDOMONAS* STRAINS.

The storage of environmental and standard strains was on nutrient agar slopes refrigerated (4°C) for up to 3 months. For long term storage aqueous glycerol solution (400 µl, 50% v/v) was added to an overnight culture (1ml) and mixed. The samples were then kept frozen (-20°C) for approximately a year. To resuscitate stored cultures, samples were thawed and inoculated onto a nutrient agar plate then incubated (1 or 2 days, 25 - 30°C). After incubation a single colony was streaked to check for colony purity on a fresh nutrient agar plate.

2.3 BACTERIAL VIABILITY.

Bacterial viability expressed as colony forming units (CFU ml^{-1}) was calculated using a serial dilution method. The culture (0.1ml) to be quantified was 10-fold serially diluted in maximum recovery diluent (0.9 ml). An aliquot (10 µl) of each dilution was spotted on to the surface of an appropriate agar plate. The dilution spots were allowed to dry and the agar plates incubated (18 hours, 30°C). Each serial dilution was tested in triplicate and an average taken.

2.4 STANDARD BACTERIAL STRAINS

ORGANISM	SOURCE	CODE
Ps aeruginosa	NCTC	10332
Ps. aeruginosa	NCIMB	10545
Ps. fluorescens	NCTC	10038
Ps testeroni	NCTC	10698
Ps alcaligenes	NCTC	10367
Ps reptilovora	IFR	461
Ps acidovorans	NCTC	10683
Ps putida	Unilever	3288
Shewanella putrefaciens	NCTC	10736
Alcaligenes faecalis	NCTC	11953
Acinetobacter junii	NCTC	12153
Flavobacterium breve	NCTC	11099
Escherichia coli	Lab. Strain	FSAC EJ1A

NCTC = National Collection of Type Cultures

NCIMB = National Collection of Industrial and Marine Bacteria

IFR = Institute of Food Research, Norwich

2.5 BIOCHEMICAL TESTS

Catalase: A spot of freshly grown test culture in broth was placed into a sterile bijoux bottle and hydrogen peroxide (1 ml, 5-10%v/v) was added. The evolution of gas indicates catalase activity.

Oxidation-fermentation: Hugh & Leifson medium was used with glucose as the substrate as described by Cowan & Steel (1974).

Gram stain: A small colony was emulsified in diluent, smeared onto a glass slide and allowed air dry. The sample was heat fixed by passing it through a blue flame. The Gram staining was carried out by immersing the slides in a series of dyes that were washed off in water before the application of the next reagent (Collins & Lyne, 1995). Methyl violet (0.5% w/v) for 1 minute; Lugol's iodine solution (2g potassium iodide in 20 ml water, with 1g ground iodine) for 30 seconds; alcohol (95% ethanol) for 1 minute and carbol fuschin (1% w/v) for 30 seconds.

Motility: Young broth cultures of the test samples grown at 25°C and examined in a "hanging drop" on a slide using a high power dry objective and reduced illumination.

A single colony of each isolate was picked using a cocktail stick which was stabbed just below the surface of the motility agar. The plates were incubated at 25°C for 24 hours. Motile organisms migrate throughout the medium, which becomes turbid; growth of the non-motile organisms is confined to the stab inoculum.

Oxidase test: Filter paper was soaked with freshly prepared 1% aqueous tetramethyl-ρ-phenylenediamine dihyrochloride. Individual colonies were picked with sterile plastic loops from nutrient agar plates and smeared onto the damp filter paper. The development of a blue colour within 10 seconds was recorded as a positive result. A *Ps. aeruginosa* and *E.coli* strains were used as positive and negative controls respectively.

Poly-β-hydroxybutyrate accumulation: Accumulation of PHB can be seen by phase contrast microscopy and confirmed by staining with Sudan black as described by Cowan & Steel (1974).

2.6 SOLUTIONS AND REAGENTS USED:

Buffer 1: 5X stock: maleic acid (500 mM, Sigma) and NaCl (150 mM, BDH) dissolved in reverse osmosis water and adjusted to pH 7.5 with sodium hydroxide pellets (BDH).

Buffer 2: 10X stock: Blocking reagent (Boehringer Mannheim) dissolved in 1X buffer 1 (10 % w/v) and stored at -20°C. On use the stock solution was diluted with 1X buffer 1 (1% w/v) and stored at 4°C.

Buffer 3: Tris-HCl (100 mM, pH 9.5), NaCl (100 mM) and $MgCl_2$ (50 mM)

Carbonate buffer: $NaHCO_3$ (0.2M) and Na_2CO_3 (80mM) pH9.2

Coating buffer: Coating buffer is used to coat microtitration plates with bacterial cells and includes Na_2HPO4 (13.4g), NaH_2PO4 (0.8g), methyl glyoxal (3.0ml, 0.3% v/v) and reverse osmosis water (1 l) pH 8.0

Coomassie Blue solution: methanol (50%), Coomassie Brilliant Blue R (0.05%), acetic acid (10%), reverse osmosis water (40%). The brilliant blue was dissolved in methanol before adding acetic acid and water.

CTAB/NaCl solution: NaCl (4.1 g, BDH) was dissolved in reverse osmosis water (80 ml) to which was added CTAB (cetyltrimethylammonium bromide)(10 g) before heating (65°C) to dissolve. The final volume was adjusted to 100 ml with reverse osmosis water before autoclaving (121°C, 15 min).

Destaining solution: Methanol (50%), acetic acid (10%), reverse osmosis water (40%).

EDTA 0.5 M: $Na_2EDTA.2H_2O$ (Sodium ethylenediamine tetraacetate acid dihydrate) (186.1 g) (Sigma) was dissolved in reverse osmosis water (700 ml). The pH (8.0) was adjusted with sodium hydroxide (10 M), and the solution was made up to a final volume of 1l with reverse osmosis water before autoclaving (121°C, 15 min).

Ethidium bromide solution (0.5mg ml^{-1}): ethidium bromide (50 mg, Sigma) was dissolved in reverse osmosis water (100 ml).

Loading buffer: EDTA (0.2 M) and glycerol (50% v/v) adjusted to pH 8.5 to which bromophenol blue (0.05% w/v) was added.

Milk Blocking buffer: PBST with skim milk powder (5%)

NaCl 5 M: NaCl (29.2 g) (BDH) was dissolved in reverse osmosis water (100 ml) and autoclaved (121°C, 15 min).

NaOH 0.4 M: NaOH pellets (16 g)(BDH) was dissolved in reverse osmosis water (1 l) and autoclaved (121°C, 15 min).

PBS (phosphate buffered saline) is a general diluent made with the addition of NaCl (8g), KH_2PO_4 (0.2g), Na_2HPO_4 (1.14g), KCl (0.2g) to 1l of reverse osmosis water.

PBST (phosphate buffered saline with Tween): This general diluent for antibodies or samples is made by mixing together NaCl (8g), KH_2PO_4 (0.2g), Na_2HPO_4 (1.14g), KCl (0.2g), Tween 20 (0.5ml) and Kathon (1.0ml) in reverse osmosis water (I l). The pH was adjusted to 7.4.

PBST +4% Tween: PBST with the addition of 4% Tween 20.

RNase A: RNase A (1 mg ml^{-1})(Sigma) was dissolved in TE buffer and boiled (10 min) to inactivate DNase activity.

Sample buffer (protein): Consisted of SDS (2%, w/v), Tris (62.5 mM pH 6.8), glycerol (0.1%, v/v), β-mercaptoethanol (0.05%, v/v) and Bromophenol Blue(0.05% (w/v).

SDS solution (10% w/v): SDS (Sodium dodecyl Sulphate) (10 g) (Sigma) was dissolved in reverse osmosis water (100 ml) and autoclaved (121°C, 15 min).

Sodium acetate 3 M: sodium acetate trihydrate (40.8 g) (Sigma) was dissolved in reverse osmosis water (100ml) and the pH adjusted to 4.8 with acetic acid (3 M) and autoclaved at 121°C for 15 min.

SSC 20X stock: NaCl (87.7 g) (BDH) and sodium citrate dihydrate (0.3 M) were dissolved in reverse osmosis water (500 ml), adjusted to pH 7.0, and autoclaved (121°C, 15 min).

TAE buffer 50X stock: Tris base (242 g)(Sigma), glacial acetic acid (57.1 ml) and $Na_2EDTA.2H_2O$ (37.2 g) were made up to 1 l with reverse osmosis water and autoclaved (121°C, 15 min).

TE buffer: Tris-HCl (10 mM, pH 9.5), EDTA (1 mM , pH 8.0) was adjusted to pH 8.0 and autoclaved(121°C, 15 min).

Transfer buffer (10X stock): Tris base (0.25 M), glycine (1.92M) and SDS (0.1% w/v) was dissolved in reverse osmosis water (1 litre). The stock solution was sterilised by autoclaving (121°C, 15 min) and a working solution made by diluting 1X at the time of use with an addition of methanol (20% v/v). The pH should be approximately 8.3.

Tris-HCl 1M: Tris-HCl (121 g) (Sigma) was dissolved in reverse osmosis water (800 ml), adjusted to pH 9.5, made up to a final volume of 1 l and autoclaved (121°C, 15 min).

2.7 PREPARATION OF POLYCLONAL ANTISERA

New Zealand White rabbits were immunised with approximately 10^9 cfu/ml (1ml) of whole, pasteurised (70°C, 10 min), exponential cells. The inoculum was emulsified in Freund's complete adjuvant (1:1, v/v) and given as subcutaneous injections on the back. Subsequent booster immunisations (1ml) were given at intervals of at least 1 month with Freund's incomplete adjuvant. Blood samples (10ml) were taken from the marginal ear vein between 9 and 14 days after giving booster injections. Following centrifugation (2000 x g, 10 min), the plasma was aspirated and stored at -20°C.

2.8 DETERMINATION OF PROTEIN CONCENTRATION

2.8.1 BCA PROTEIN ASSAY

The BCA Protein Assay kit (Pierce, Illinois) was used for the spectrophotometric determination of protein concentration. The assay is based on the Biuret reaction where protein reduces Cu^{2+} in an alkaline environment to Cu^{1+}. BCA (bicinchoninic acid) reacts with Cu^{1+} to form a purple product that exhibits a strong absorbance at 562nm (Figure 2.1).

A set of protein standards of known concentration was prepared by diluting bovine serum albumin in PBST. Each standard (0.1ml) or test sample was pipetted into labelled tubes. An aliquot (0.1ml) of PBST was used as a blank. To each tube working reagent (2ml) was added and mixed. All tubes were then incubated (37°C, 30 min) and allowed to cool down to room temperature. The absorbance of each tube was measured (562nm) against a water reference (as per manufacturer's instructions). The results from the standards were used to construct a standard curve by plotting the blank corrected absorbance against protein concentration. The standard curve was then used to determine the protein concentration for each unknown.

2.8.2 SPECTROPHOTOMETRIC ESTIMATION OF PROTEIN CONCENTRATION

Protein concentration was estimated by reading the absorbance of protein solutions at 280nm(Lambda 9, Perkin Elmer), using 1cm quartz cuvettes (Starna Ltd, Romford UK). An appropriate buffer was used as a blank. The protein concentration was calculated using Beer Lambert's equation (Equation 1)

Equation 1.1 Beer Lambert's Equation

$$A = E\,c\,l$$

$$c = \frac{A}{E\,l}$$

$$c = \frac{A}{1.4}$$

Where A = absorbance at 280 nm, E = protein extinction coefficient, (1.4), c = protein concentration, mg/ml and l = path length (1 cm)

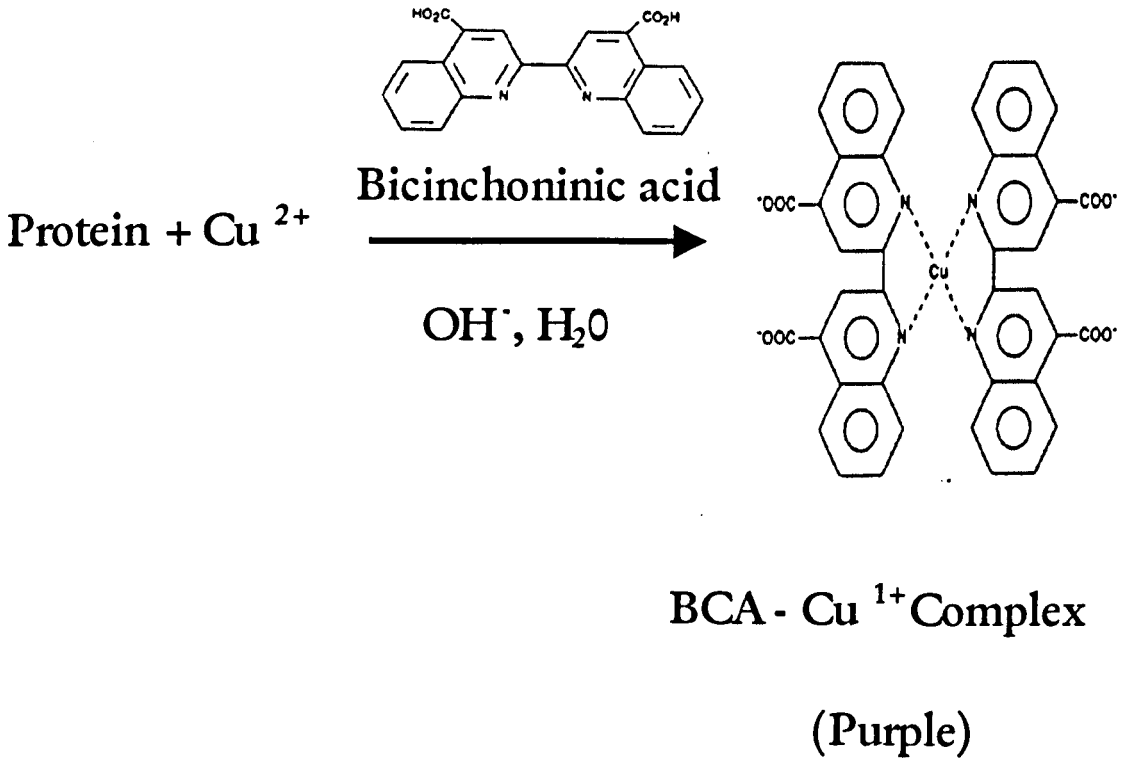

Figure 2.1 BCA Protein Assay Reaction

2.9 COATING OF MICROTITRATION PLATES

Late exponential vegetative cells were harvested by centrifugation and resuspended in coating buffer (Section 2.6) to give a 2-5μg ml^{-1} solution (estimated using BCA protein assay, Section 2.8.1). An aliquot (200μl) was added to each well of the microtitration plate and either incubated overnight at 4°C or at 37°C for 2-3 hours. The coated plates were washed (x5)(using Wellwash 5000, Life Sciences International UK) in PBST and any unreacted binding sites blocked by adding milk blocking solution (300μl) to each well. Each plate was incubated and washed as before, air-dried and stored desiccated in the dark at room temperature.

2.10 ANTIBODY TITRES

For each serum ten- fold dilution series were made in PBST. Duplicate volumes (200μl) were added to the wells of an appropriately cell-coated microtitration plate. The plates were incubated with lid (1 hour, 37°C), then washed in PBST (X5). Diluted anti-rabbit horseradish peroxidase labelled antibody (IgG-HRP, 1:1000 v/v in PBS) was added to each well (200μl). The plates were incubated with the lid (1 hour, 37°C), washed five times in PBST and developed by the addition of TMB (3,3', 5,5'-tetramethylbenzidine) (200μl, 0.1mg/ml w/v) substrate. The reaction was stopped with 1M sulphuric acid (50μl) and the microtitration plates read automatically with a microplate reader (MR5000, Dynatech) at 450nm.

2.11 DOT BLOT

Overnight cultures of *Pseudomonas* strains were washed twice in saline (0.85% NaCl, 3000g for 1 min) and resuspended in PBST to an optical density of 0.2 at 600nm. The washed cell suspensions (10μl) were spotted in duplicate onto a nitro-cellulose membrane (Hybond-C, Amersham) air dried, blocked with PBST + 4% Tween (or PBST with 5% skim milk powder, 10ml per filter) and incubated shaking (1 hour, room temperature) in the presence of the test or control antisera (1/1000 - 1/5000v/v). The filters were washed twice (PBST for 5 min) incubated with anti-rabbit IgG-HRP added (room temperature, 30 min). After further washing (PBST for 5 min) the colour was developed, using DAB (3,3 diaminobenzidine, Sigma)(10 ml, 0.5mg/ml w/v). The ability of the

antisera to bind to each isolate was assessed by visually comparing the colour of each colony spot stained with the test antiserum and with non- immune serum.

2.12 COMPETITIVE ELISA

Cell suspensions of test and standard cultures (100µl) were added to coated wells on a microtitration plate in duplicate followed by a limited amount of the antisera (100µl, 1/5000v/v). After incubation (37°C, 2 hours) the plates were washed in PBST (X5) and anti-rabbit IgG-HRP (100µl, 1/1000v/v) was added. The plates were incubated (1 hour, 37°C) and washed in PBST (X5) prior to the development of colour with TMB substrate (100ul, 1-5 min). The reaction was stopped with sulphuric acid (50µl, 1M) and the absorbance (450nm) was determined using a microplate reader.

PBST was used as a control and duplicated up to 10 times on each microtitration plate. The mean PBST was calculated and the standard deviation calculated. A positive reaction was defined as displacement greater than two times the standard deviation of the mean PBST control.

2.13 PURIFICATION OF POLYCLONAL ANTIBODIES

An aliquot (2ml) of antiserum was diluted with two volumes of phosphate buffered saline containing NaCl (3M) and glycine (1.5M)(pH 8.9). The sample was microfuged to remove large cellular debris and then filtered through a 0.22µm filter (Millipore). The filtered sample was injected onto a protein A fast flow Sepharose column (Pharmacia Biotech, Sweden) which was equilibrated in start buffer (3M NaCl, 1.5M glycine, pH8.9). Bound IgG was eluted with citric acid (0.1M) at pH 6 and pH 4. The pH of the antibody-containing fractions was readjusted to 7.4 with 1M Tris.

2.14 DIRECT LABELLING OF ANTIBODIES

2.14.1 BIOTIN

Samples of purified antisera were dialysed overnight against 0.1M NaHCO3, pH 8.0, with three changes of buffer. The antibody concentration was then determined spectrophotometrically (Section 2.1.2) and adjusted to 1mg/ml. If the protein concentration was too low the antibody sample was concentrated using a Centriprep 30 concentrator (Amicon Inc., USA) and if the concentration was too high then it was diluted in the dialysis buffer. A stock of sulfo-N-hydroxysuccinimide- LC (NHS-LC) biotin ester (1mg/ml) was made in dimethylsulphoxide (DMSO). The ester was added at a concentration of 50μg per mg of protein present and the reaction was allowed to proceed at room temperature for 1 hour. The biotinylated antibody was then purified on a PD-10, Sephadex G-25 column (Pharmacia) to remove excess NHS-LC-biotin, using the dialysis buffer as both the equilibration and elution buffers. The eluted fractions were collected and the concentration of protein containing fractions determined spectrophotometrically (Section 2.1.2)

2.14.2 FLUORESCEIN ISOTHIOCYANATE (FITC)

Samples of purified antisera were dialysed overnight against carbonate buffer (2.6) with three changes of buffer and the protein concentration determined spectrophotometrically (Section 2.1.2). A stock of FITC (1mg/ml, Sigma) in DMSO was prepared. FITC stock was added to the antibody solution, in the dark, at a concentration of 100 μg per mg of protein present. Conjugation was allowed to take place at room temperature for 3 hours. Unreacted FITC was removed from the samples by running them on a PD-10 column that was previously equilibrated and subsequently eluted with phosphate buffered saline with sodium azide (0.1% w/v). The eluted fraction containing the conjugated antibody was stored at 4°C and protected from light.

2.15 PROTEIN GELS

2.15.1 PREPARATION OF TOTAL CELL PROTEINS.

Total cellular proteins were extracted from bacterial culture (10 ml) grown to stationary phase. The cells were harvested by centrifugation (3000 x g, 15 min)(MSE rotor, Centaur 2), resuspended in phosphate buffered saline (PBS)(1 ml) and transferred to an Eppendorf tube. The cells were repelleted by centrifugation (13000 x g, 3 min)(Heraeus Sepatech microfuge) and resuspended in PBS (100 μl) and 2X sample buffer (100μl)(Section 2.6).

The mixture was boiled (5 min) in a water bath for cell lysis. The insoluble material was pelleted by centrifugation (13000 x g, 4 min) and the supernatant was transferred into a clean Eppendorf tube. The sample could then be loaded onto a SDS- polyacrylamide gel.

2.15.2 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS.

Proteins were analyzed using a BioRad minigel system according to the manufacturer's protocol. Approximately 20 μg of total cell protein was loaded into each well. Standard low molecular weight markers (BioRad) were loaded at 10 μg per lane. The gels were electrophoresed at 100 v – 200v.

2.15.3 STAINING AND DESTAINING OF GELS.

After the gel was run, the proteins were either immobilised onto a membrane or stained to see the total protein profile using coomassie blue staining solution (Sambrook *et al.*, 1989). Typically the gel was stained in coomassie blue solution (1 hour) and then destained, shaking with frequent changes of destaining solution (Section 2.6) until the background became clear.

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2.16 LPS

Preparation of lipopolysaccharide was achieved using proteinase K (Sigma). An overnight culture (10ml) was harvested and washed in PBS. The cells were resuspended in PBS to give an absorbance at 600nm of 0.5-0.6. The cell suspension (1.5ml) was centrifuged (10000 x g, 3 min; Biofuge 13, Heraeus sephatech). The pellet was resuspended in SDS PAGE sample buffer (50 μl; 2% SDS, 0.1% mercaptoethanol, 10% glycerol, 0.002% bromophenol blue, 50 mM Tris-HCl pH 6.8). The mixture was boiled (100°C, 10 min). Proteinase K solution (2.5mg proteinase K per ml SDS PAGE sample buffer) was added and the mixture heated (60°C, 60 min). The extracted LPS was then run on a SDS PAGE gel (12%).

The bands were visualised using silver staining. Silver staining was achieved by using a Pharmacia Biotech (St. Albans, UK) silver staining kit as per the manufacturer's instructions..

2.17 WESTERN BLOTTING

The protein gel was soaked in transfer buffer (15-30 min). Six pieces of 3 MM Whatmann filter paper and one piece of PVDF membrane (BioRad) were cut to the size of gel. A sandwich was prepared with membrane facing cathode and gel facing anode surrounded by three filter papers on each side. The trapped air bubbles were rolled out with the help of a clean pipette. The sandwich was kept in between the foam sheets and set in apparatus (Electroblot Apparatus; BioRad). The inner compartment was filled with 3 litres of transfer buffer. The electroblotting was achieved by running the apparatus at 80 v for 2 hours or 30 v overnight while cooling with running tap water. After blotting the membrane was stained with Ponceau-S stain (Sigma) to check blotting efficiency.

2.17.1 WESTERN BLOTTING AND DETECTION.

The membrane was blocked overnight (4°C) in milk blocking buffer. The membrane was then washed in PBST (x3, 5 min). The primary antibody was diluted (1:1000- 1:5000 v/v) in milk blocking buffer. The membrane was then incubated, shaking in diluted primary antibody (2 hours, room temperature). The membrane was washed three times with PBST (x3, 5 minutes, room temperature). The secondary antibody (anti-rabbit horse- radish peroxidase conjugate; Sigma) was diluted (1:1000 v/v) in PBST. The membrane was incubated, shaking in diluted secondary antibody (2 hours, room temperature). The membrane was again washed as before and the colour developed using DAB (diaminobenzidine, Sigma) as per manufacturer's instructions. The colour development was stopped using tap water.

2.18 DNA EXTRACTION

Preparation of genomic DNA was carried out according to the protocol outlined by Ausubel *et al.* 1990 using a cetyltrimethylammonium bromide (CTAB) extraction procedure. Cultures were grown statically overnight (30°C) in half strength BHI and harvested (1.5ml) by centrifugation (Biofuge 13, Heraeus sephatech) at 15000 x g for 2 minutes. The bacterial pellet was washed twice in saline (0.85% NaCl) and resuspended in 567µl of TE buffer. Freshly prepared lysozyme (30µl, 40mg/ml) was added and the tubes were incubated (30 min, 37°C). SDS (30µl, 10% w/v) and Proteinase K (20 mg/ml, Sigma) was added and the mixture incubated (1h, 37°C). Sodium chloride (100µl, 5M) and CTAB solution (warmed to 65°C). An equal volume of chloroform/isoamyl alcohol (24:1, v/v) was added, mixed and centrifuged (4minutes, 15000 x g). The upper aqueous layer

was removed to a fresh tube and 0.6 volumes of isopropanol were added and the tube placed at -20°C to precipitate the DNA (~20min). Centrifugation (15000 x g, 10min) pelleted the DNA which was washed twice with 70% ethanol. The supernatant was discarded and the DNA pellet was air dried before being resuspended in 50 -100µl of TE buffer.

2.19 EXAMINATION OF DNA BY AGAROSE GEL ELECTROPHORESIS.

In order to quantify and analyse DNA samples all preparations were examined by gel electrophoresis on 0.8 % agarose gels (1% agarose was used for the examination of small fragments of DNA when necessary). Gels were prepared by heating the desired quantity of agarose (Flowgen) in 1X TAE buffer until all particles of agarose had dissolved. On cooling (to approximately 55°C), ethidium bromide solution (final concentration of 0.5 µg ml^{-1}) was added and the molten agarose poured into a gel tray (Anachem) and left to set. Gels were then immersed in Anachem Origo horizontal tanks containing 1X TAE buffer. DNA samples to be visualised were mixed with 0.2 volumes of loading buffer and loaded into the gel wells. A voltage (60-80 V) was then applied until the desired resolution of DNA bands was achieved. For the estimation of bands sizes λ *Hind* III (0.5 µg)molecular weight marker (MBI Fermentas) or a 100 bp ladder marker (Pharmacia) was used. DNA bands were visualised with an UV transilluminator emitting at 313 nm, and photographed using a Mitsubishi video copy processor, or with an Olympus OM-2 35 mm camera.

2.20 PHENOL/ CHLOROFORM EXTRACTION OF DNA.

In order to inactivate restriction enzymes or to remove excess debris from DNA preparations, samples were treated with phenol/chloroform as described by Sambrook *et al.*, (1989). 1 volume of phenol was added to the DNA sample and shaken vigorously. The resultant emulsion was then spun (13 000 x g, 15 min, Heraeus Sepatech microfuge) and the upper layer transferred to a clean Eppendorf tube. One volume of chloroform/ isoamylalcohol (24:1) was then added, the sample shaken vigorously and centrifuged (13 000 x g, 10 min). The upper layer was removed to a clean Eppendorf tube and chloroform/ isoamyl alcohol extracted as before. The DNA was then precipitated by the addition of 100 % ethanol (2.5 volumes) and 3 M sodium acetate (0.1 volume, -20°C for 20 min). The precipitated DNA was centrifuged (13 000 x g, 15 min) and the pellet

washed twice with 70% ethanol. The DNA pellet was allowed to air dry before resuspending in TE buffer (100µl).

2.21 RIBOTYPING

2.21.1 CLEAVAGE OF DNA WITH RESTRICTION ENZYMES.

Chromosomal digests of DNA (1-5 µg) were carried out using 2U of endonuclease with the appropriate buffers (Pharmacia) under the conditions recommended by the manufacturer. Typically, digests were carried out at 37°C for 2 hours. If the digestion of chromosomal DNA was incomplete after 2 hours then the incubation period was extended until complete digestion was attained. 1 µl of RNase A solution (1 mg ml^{-1}) was included in reaction mixtures where appropriate.

2.21.2 RIBOPROBE PREPARATION (REVERSE TRANSCRIPTION)

The riboprobe (cDNA) was prepared from 16S and 23S rRNA using reverse transcriptase. The following were added to a sterile Eppendorf tube: 10x hexanucleotide mixture (2µl, Boehringer Mannheim), 10x Wachsmuth reaction buffer (2µl, 500mM Tris-Cl pH 8.3, 60mM $MgCl_2$, 400mM KCl), *E.coli* 16S and 23S rRNA (0.25µl (4µg/ml), Boehringer Mannheim) and sterile reverse osmosis water (14.25µl). The mix was heated (68°C, 5 min) and then slowly cooled to room temperature. The following were added: 10x dNTP labelling mixture (2µl, containing dig-dUTP) (Boehringer Mannheim) and AMV reverse transcriptase (1.5µl, 20U/µl) (Boehringer Mannheim) and mixed. After incubation (42°C, 1 hour), 4M lithium chloride (2µl) and cold ethanol (50µl) were added and the probe then precipitated (-20°C for 1 h). After centrifuging (13000 g, 15 min, Biofuge A Heraeus Sepatech,), the supernatant was removed and the pellet washed with 70% ethanol. The pellet was air dried and resuspended in TE (20µl, pH 8) and hybridization solution (10ml, 5x SSC, 1% blocking reagent (Boehringer Mannheim), 0.1% w/v sarkosyl, 0.02% w/v SDS). The probe was stored at -20°C for up to a year.

2.21.3 MARKER-PROBE PREPARATION (RANDOM PRIMER EXTENSION)

The marker probe was prepared to allow both the *Lambda/Hind*III and 1Kb ladder markers to be visualised. The following were added to a sterile Eppendorf tube: 2-3µg

1Kb ladder, 0.5g *Lambda/Hind*III and 12µl SDW. After vortexing and a brief spin (Biofuge A Heraeus Sepatech, 13000 g), the Eppendorf tube was boiled (10 min) and then cooled on ice (5 min). The following were then added: hexanucleotide mix (2µl, Boehringer Mannheim), dNTP labelling mix (2µl, Boehringer Mannheim) and Klenow enzyme (1µl, 2U/µl, Boehringer Mannheim). The reaction was performed (1 hour, 37°C) and then the reaction stopped using 0.5M EDTA (0.8µl, pH 6). The probe was stored at -20°C in hybridisation solution (10ml) containing the riboprobe.

2.21.4 SOUTHERN HYBRIDISATION.

Southern hybridisation was based on the method described by Ausubel *et al.* (1989). Chromosomal DNA (~10 µg) was digested with the appropriate restriction enzyme, electrophorised in an agarose gel (1.0 %) overnight at 15 V, and photographed. The DNA was then transferred on to a positively charged nylon membrane (Hybond N^+, Amersham International PLC) by vacuum blotting. The vacuum blotting apparatus (Hybaid Ltd) was set up as follows; 2 sheets of Whatman 3 MM filter paper were soaked in 0.4 M NaOH and placed on the vacuum blotter, the nylon filter (cut to +1 cm the size of the gel) was also soaked in the 0.4 M NaOH and placed on the 2 sheets of pre-soaked filter paper. All air bubbles were removed and the filter paper and nylon membrane covered with a rubber template so that the membrane area was exposed. The agarose gel to be blotted was then placed onto the surface of the nylon membrane so that the outer edges of the gel partially overlapped with the rubber template and no areas of the membrane were exposed ensuring that a vacuum could then be generated. Securing the lid and connecting to a vacuum pump (Hybaid Ltd) sealed the apparatus. The surface of the gel was flooded with 0.4 M NaOH and a vacuum applied for 1 hr. The position of the gel wells was marked using a syringe needle and the gel removed. The nylon membrane was then washed for 1 min in 2X SSC and dried between 2 sheets of Whatman 2 MM filter paper.

2.21.5 PRE-HYBRIDIZATION AND HYBRIDIZATION

Prehybridisation of the membrane was carried out by sealing the membrane in a plastic stomacher bag or roller tube with 15 ml of DIG Easy Hyb™ (Boehringer Manheim) and incubated (1 hour, 42°C) in an incubator or water bath. The DNA probe was heat denatured by boiling in a water bath (10 min) and added to the stomacher bag or roller tube containing the prehybridised membrane. The bag or roller tube was then resealed and hybridisation carried out overnight in a water bath (42°C).

2.21.6 POST-HYBRIDIZATION AND DETECTION

After hybridisation overnight the remaining probe was removed from the sealed bag or roller tube and retained for future use at -20°C. The membrane was washed twice (15 min) with a solution of 2X SSC and 0.1% SDS at room temperature. This was followed by two washes (15 min at 65°C with a solution of 0.1X SSC and 0.1 % SDS). The membrane was then equilibrated in 1X buffer 1 (1 min) and transferred to 1X buffer 2 (blocking reagent)(30 min, room temperature, shaking).

2.21.7 COLORIMETRIC DETECTION

Anti-DIG alkaline phosphatase Fab fragments (Boehringer Mannheim) was diluted 1:5000 in 1X buffer 2 (30 ml) and the membrane sealed in a bag with this solution and then gently shaken (30 min, room temperature). The antibody solution was removed and the membrane washed twice (15 min) in 1X buffer 1 to remove excess antibody. After equilibration in buffer 3 (2-3 min) the regions of the membrane which had hybridised to the probe were detected colormetrically. Detection was carried out in the dark in a sealed bag containing colour solution (10 ml, 45µl (75mg/ml) nitroblue tetrazolium salt (NBT) in dimethylformamide (DMF) (Boehringer Mannheim), 35µl (50mg/ml) 5-bromo-4-chloro-3-indolyl-phosphate toluidinium salt (BCIP or X-phosphate) (Boehringer Mannheim) in DMF, 10ml buffer 3 (section 2.6)). When a clear signal was apparent washing the membrane in TE (pH8, 5 min) terminated the reaction and the membrane blotted and air dried.

2.22 ARDRA POLYMERASE CHAIN REACTION.

Amplified ribosomal DNA restriction analysis (ARDRA) involves the amplification of the 16S rRNA gene. The gene product (~1Kbp) is then digested with restriction enzymes.

20 base pair rDNA primers: Forward 5' GCT CAG ATT GAA CGC TGG CG 3'
Reverse 5' ACA TTT CAC AAC ACG AGC TG 3'

Each primer was used at a concentration of 1µg/µl (Gensis synthesiser, Cambridge, UK)

The polymerase chain reaction (PCR) (Mullis *et al.*, 1986) was used for the amplification of specific regions of DNA. PCR was preformed on pre-isolated DNA samples (~0.5 µg) or boiled cells (2µl) in micro Eppendorf tubes. Each tube contained 100 pM of each primer

(forward and reverse), 25 mM each of dATP, dCTP, dGTP and dTTP, 2 mM $MgCl_2$ and 10X *Taq* DNA polymerase buffer (5 µl, Perkin Elmer) made up to a total volume of 50 µl with sterile reverse osmosis water. PCR was performed in a Techne PHC-3 thermal cycler, after an initial cycle at 94°C for 10 min 1U of *Taq* DNA polymerase (Perkin Elmer) was added. Following PCR 8 µl of product was mixed with 3 µl of loading buffer and electrophoresed in a 1.5% agarose gel containing ethidium bromide (0.5mg/ml) in 1x TAE running buffer at 70V for 2-3 hours. Size markers were also included on the gel.

2.22.1 PCR CONDITIONS

1 cycle	94°C	10min	(hot start)
30 cycles	94°C	30 seconds	(melting/strand separation)
	50°C	60 seconds	(primer annealing)
	72°C	60 seconds	(extension/polymerisation)
1 cycle	72°C	10 minutes	

2.22.2 TOUCHDOWN PCR CONDITIONS

1 cycle	94°C	10min	
15 cycles	94°C	30 seconds	
	65°C	60 seconds	(temperature reduced 1°C after each cycle)
	72°C	60 seconds	
15 cycles	94°C	30 seconds	
	50°C	60 seconds	
	72°C	60 seconds	
1 cycle	72°C	10 minutes	

Chapter 3

ANTIBODY PRODUCTION AND CHARACTERISATION

3.1 INTRODUCTION

Within the host each immunoglobulin molecule is capable of binding specifically to the antigen which triggered the immune response. *In vitro* the specific binding properties of antibodies can be exploited, under the appropriate conditions, in a variety of ways.

The production of both monoclonal and polyclonal antibodies is well established and because of this it is often thought of as a fully characterised and predictable event. This however, is not the case. The epitopes of single antigens recognised by each immunoglobulin may vary greatly as does the binding affinity and conditions under which binding take place. Therefore the antibody population can be highly heterogeneous. To obtain the best antibody for a given purpose it is essential to screen for required characteristics so as to establish limitations and optimum working conditions. Even the best antibodies can perform sub-optimally if the conditions are not appropriate.

The choice of method of antibody production for this study was made with the final use of the antibodies in mind. The intention was to use the antibodies to stain the surface of bacterial cells. Using polyclonal antibodies would facilitate entire cell staining more readily than monoclonal antibodies due to the array of epitopes that should be recognised. In addition polyclonal antibodies may be quicker and easier to produce than monoclonal antibodies which can be time consuming to generate and highly labour intensive. The heterogeneous nature of antibodies in a polyclonal serum means that good binding is likely to occur in a wider range of conditions. With the polyclonal population there may be higher affinity antibodies than is usually observed in single monoclonal antibodies.

3.2 AIMS AND OBJECTIVES

The aims were to produce antibodies that were specific to defined spoilage *Pseudomonas* populations and which could be used in locational studies. Each antibody raised would be characterised in terms of titre, affinity and cross-reactivity. The nature of the epitopes that each antibody was recognising would also be investigated.

3.3 PRODUCTION OF ANTIBODIES

3.3.1 INTRODUCTION

Polyclonal antibodies raised in New Zealand White rabbits against whole pasteurised cells of *Pseudomonas fluorescens*, *Ps. putida*, *Ps. alcaligenes* and *Ps.aeruginosa* had been produced by Emma Parsons at the Institute of Food Research, Norwich prior to the commencement of this work. The *Pseudomonas* strains had been isolated from spoiled fish (*Clupea sprattus*). The sera produced had not previously been characterised. Three additional polyclonal antibodies were raised in a similar manner against *Ps. putida* (RM5), *Ps. fluorescens* (RM1; from spoiled raw milk) and *Ps.aeruginosa* (NO31; from spoiled chicken, courtesy of the Food Hygiene Laboratory, Central Public Health Laboratory, Colindale).

The antisera produced were raised against washed whole cells of *Pseudomonas* species. The washing process was non-stringent with the aim of removing media components and extra-cellular products. However, *Pseudomonas* species can produce extra-cellular polysaccharides which can be highly immunogenic (Wyatt , 1992) and so stringent washing was not used to prevent their removal. Investigations were made to find out where the antibody was binding e.g. on the cell surface, on extra-cellular polysaccharide, or on flagella. Techniques used were whole cell extract immuno-blots, scanning electron microscopy (SEM) and atomic force microscopy (AFM).

The evaluation of each of the antiserum titres was performed in a microtitration plate format to establish the most effective working dilution for each protocol. The cross-reactions and affinities were assessed using a competitive ELISA and a non-competitive dot-blot technique. In the non-competitive method a single analyte was incubated with the antibody and the total amount of binding was measured. In the competitive method the antibody concentration is limiting and antibody can bind to a choice of analytes. The staining of bacterial cells is a non-competitive process. The dot-blot method has the advantage of being able to compare the staining reactions of many different cells simultaneously though the results are not easily quantified. The results obtained by the competitive ELISA method are quantifiable and were used to complement results obtained non-competitively.

3.3.2.1 Isolation of *Pseudomonas* strains

Samples of the psychrotrophically-spoiled food (4°C, up to 13 days) were stomached and diluted in brain heart infusion broth. Aliquots of each dilution were spread plated onto *Pseudomonas* CFC agar (section 2.1.3). Typical *Pseudomonas* colonies were sub-cultured onto nutrient agar and 1 day old colonies phenotypically screened using the Gram stain, motility, oxidase and Hugh and Leifson oxidation and fermentation tests. Those strains, which were Gram negative, motile, oxidase positive and oxidative, were then screened using API 20 NE strips (BioMerieux SA, Marcy l'a Etoile, France). Strains identified as *Pseudomonas* were stored on nutrient agar slopes.

3.3.2.2 Preparation of antigen

Pseudomonas cells were grown statically overnight in BHI (30°C) and contained approximately 10^8 cells per ml, which were then pasteurised (70°C for 10 minutes in a water bath).

3.3.2.3 Immunisation Protocol

Freshly pasteurised cells were cooled and used in conjunction with Freund's adjuvant to immunise New Zealand White rabbits (as section 2.7). Freund's complete adjuvant contains dead *Mycobacterium* cells, which provoke an inflammatory response, enhancing antibody formation. The first dose contained the immunogen vigorously mixed with Freund's complete adjuvant to form an emulsion. The water-in-oil emulsion prevents the destruction of the immunogen, allows the immunogen to diffuse slowly into the tissues and prolonging the stimulus. Subsequent dosages were emulsified in Freund's incomplete adjuvant (without *Mycobacterium*) to minimise the formation of lesions at the injection sites (Wyatt, 1992).

The immunisation protocol is outlined in section 2.7 and bleeds were taken 9 -13 days after a booster immunisation, to coincide with the maximum IgG production in the rabbit (Fig. 3.1)

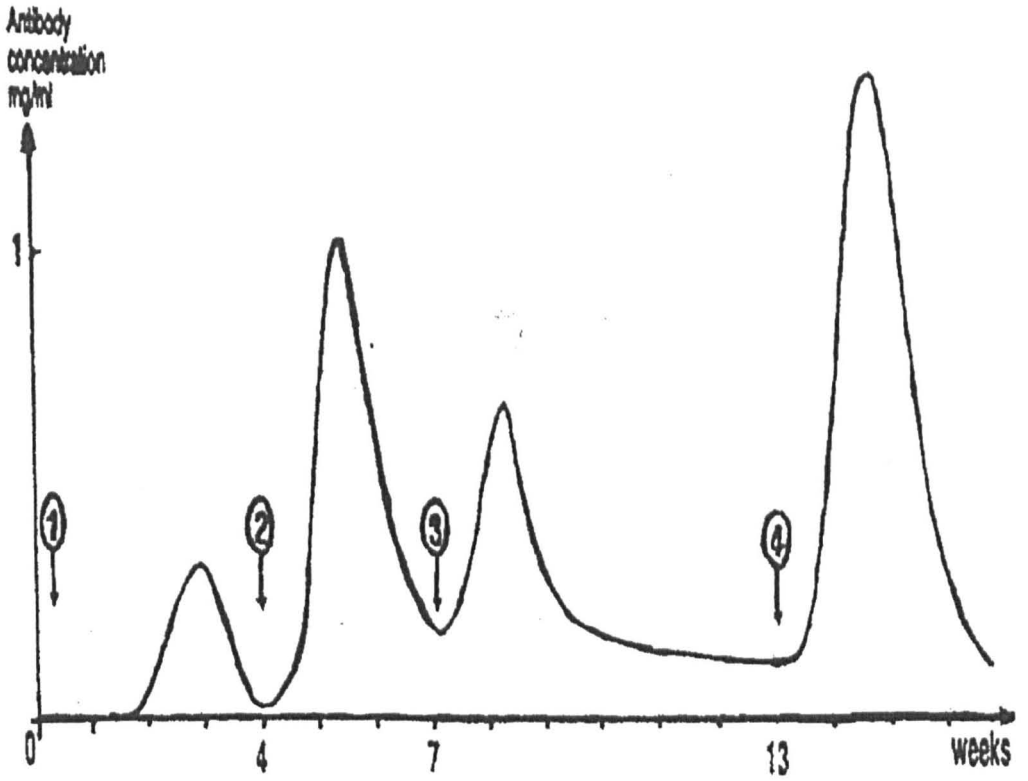


Figure 3.1 IgG Production in Rabbits

This figure shows the typical IgG response of a rabbit to variously timed immunisations with an antigen (numbered 1 – 4). Based on a diagram from Wyatt *et al.*, 1992

3.4 ANTISERUM TITRES

3.4.1 INTRODUCTION

Dilutions of each antiserum were assessed to find the optimum strength to use in a staining protocol. The sera were titred for use on cells bound to membranes and wells of microtitration plates. The bacterial antigens used to raise each antiserum are shown in Table 3.1.

3.4.2 METHOD

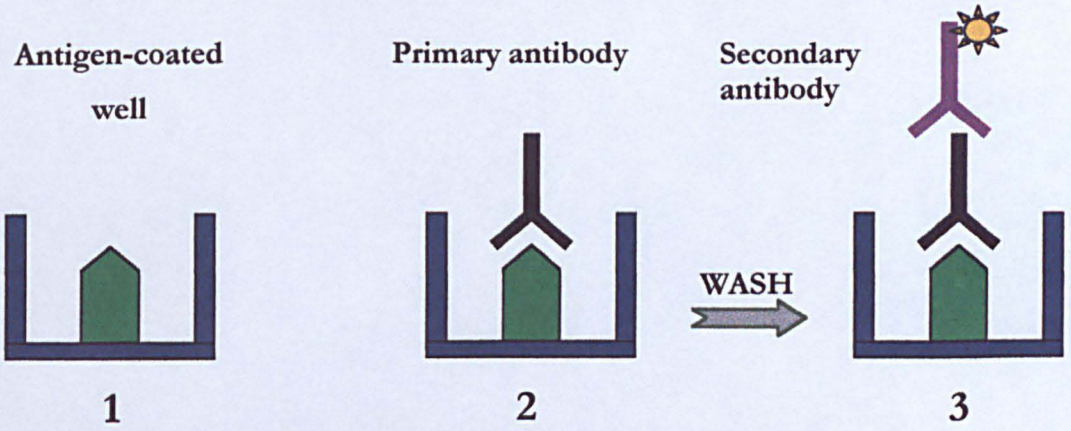
3.4.2.1 Determination of protein concentration

To standardise serum and bacterial cell suspensions the amount of protein present was assessed. Protein determinations were made using the BCA Protein Assay kit (Pierce, Rockford, USA; section 2.8.1) or spectrophotometrically (Lamba 9, Perkin Elmer; section 2.8.2). A typical BCA protein assay standard curve can be seen in Figure 3.3.

3.4.2.2 Microtitration plate titres

The evaluation of the serum titres in each bleed taken from each animal, was carried out in the inner 60 wells of 96 well microtitration plates (to overcome possible edging effects), as in sections 2.9 and 2.10. A diagrammatic representation of the immunoabsorbent assay can be seen in Figure 3.2.

Figure 3.2 Diagrammatic representation of the microtitration plate assay



1 Microtitration plates coated with a known amount of bacterial cells (antigen).

2 Dilutions of test (primary) antiserum added to wells and incubated.

3 The antibody-antigen-binding event indirectly detected and quantified using a secondary antibody conjugated to an enzyme, which in the presence of a substrate produces colour.

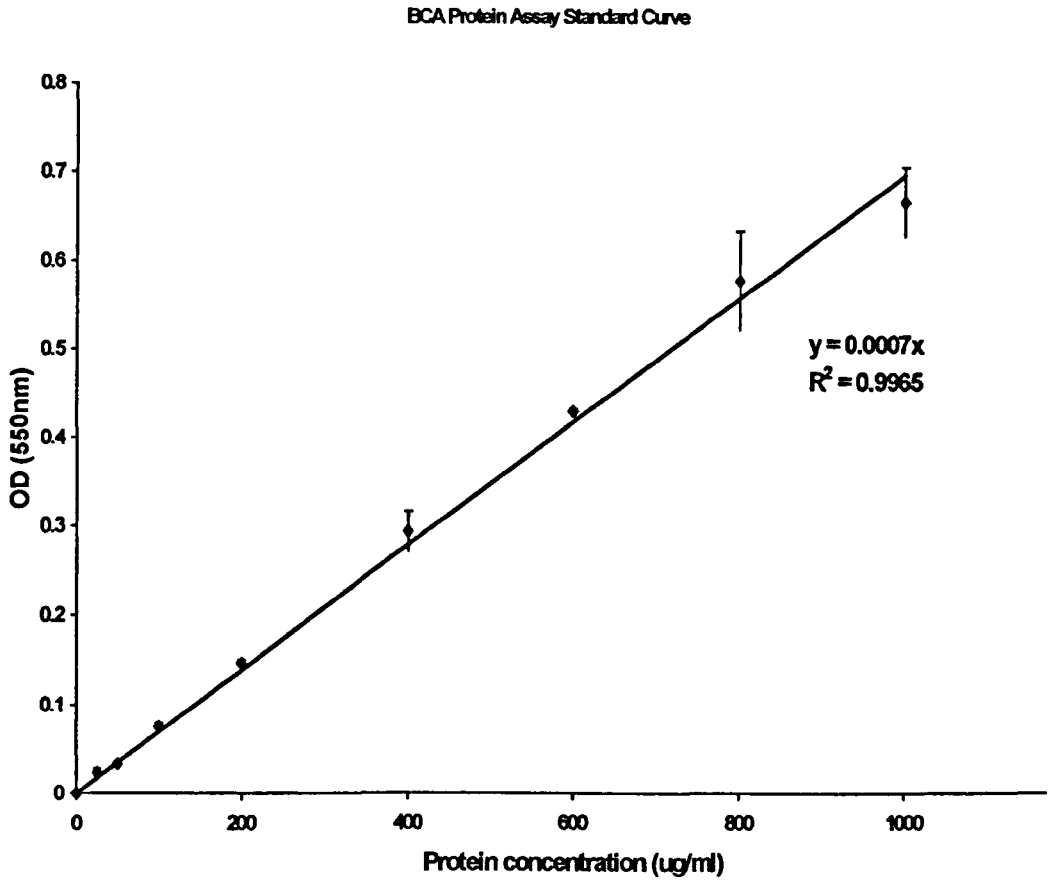


Figure 3.3 Typical BCA Protein assay standard curve

The error bars at each datum point represents the standard deviation generated from two replica determinations. R^2 = the correlation coefficient. The equation of the straight line that best fits the data points (generated by the least squares method) is also shown

CODE OF RABBITS	CODE OF ANTISERA	SPECIES USED TO RAISE ANTISERA	ISOLATE CODE OF EACH IMMUNOGEN	SOURCE OF IMMUNOGEN
R462	EDWARD	*Ps. fluorescens*	EM1, 2, 3, 27, 28, 29, 33, 35, 36	FISH
R463	EGBERT	*Ps. putida*	EM11, 12, 14, 17, 20	FISH
R464	ENTWISTLE	*Ps. aeruginosa*	EM31	FISH
R465	EAMON	*Ps. alcaligenes*	EM9	FISH
R548	XAVIER	*Ps. fluorescens*	RM1	MILK
R549	XERXES	*Ps. putida*	RM5	MILK
R551	XTRA	*Ps. aeruginosa*	NO31	CHICKEN

Table 3.1 *Pseudomonas* Isolates Used To Produce Polyclonal Antisera

Edward and Egbert antiserum (all sera named after the rabbits in which they were raised) were raised against multiple immunogens.

3.4.2.3 Dot blot titres

Standardised cell suspensions of each immunogen were fixed on to a nitrocellulose membrane and were incubated in the presence of various concentrations (10^{-2} - 10^{-5} dilutions) of the primary serum (section 2.4). Reacting immunogens was visualised using a labelled secondary antibody.

3.4.3 RESULTS AND DISCUSSION

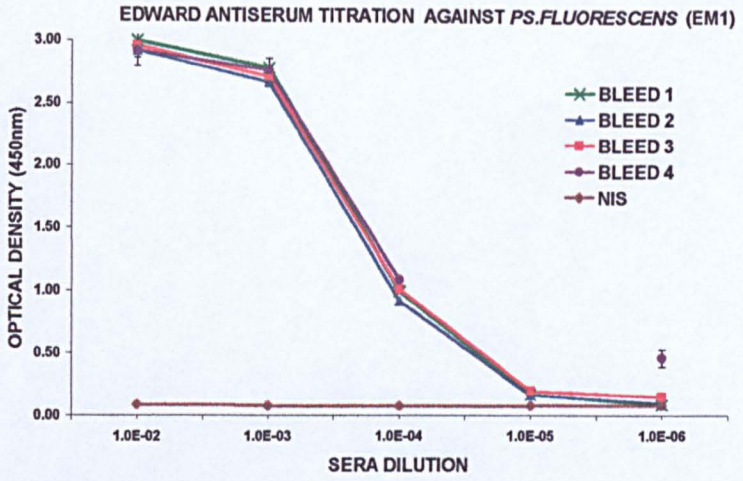
3.4.3.1 Plate titres

Typical serum titre curves are represented in Figures 3.4, 3.5, 3.6 and 3.7. Edward and Egbert antisera were raised against multiple immunogens. The titres for each of the multiple immunogens were different and broadly fell into a low titre or high titre group. Figure 3.4 shows the titres for three of the nine immunogens used to raise Edward antisera. Edward antisera showed a high titre of antibodies for *Ps. fluorescens* EM1 and EM36 whereas, comparatively, EM28 showed lower titre levels. EM 35 also showed low titre levels similar to that of EM28. EM2, EM3, EM27, EM29 and EM33 gave higher titre levels (data not shown). A similar picture was apparent for the Egbert antisera (Fig.3.5), such that EM20 and EM17 (data not shown) gave high titres and EM11, EM12 (data not shown) and EM14 gave low titres. For both the Edward and Egbert antisera bleeds 1 and 2 gave significantly lower titres than bleeds 3 and 4.

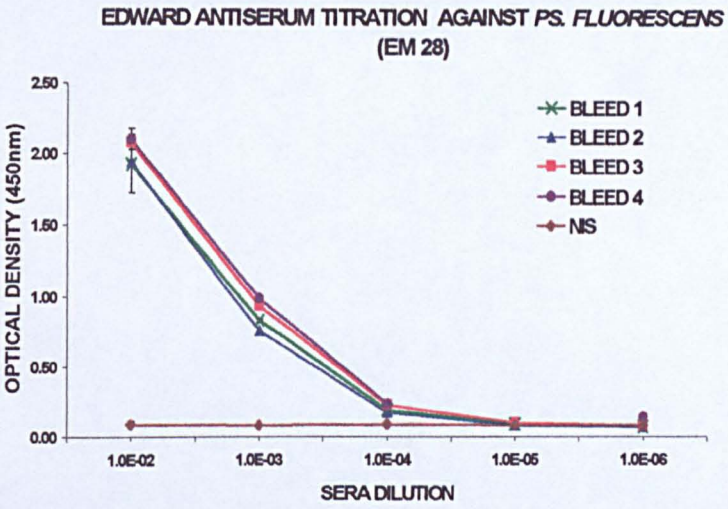
The titration curves for Eamon antisera (Figure 3.6A) showed that all bleeds produced a similar titre level (low). Each bleed from the Entwistle antisera gave a high antibody titre, although at higher serum dilutions ($\leq 10^{-4}$)a distinct difference between the titres of Entwistle bleeds 1 & 2 and those of 3 & 4 could be seen (Fig. 3.6B).

From the titration assays for the X series of antisera (Xerxes, Xtra and Xavier; Figures 3.7A, B and C respectively) the control NIS showed a high degree of non-specific binding at low dilution (10^{-2}). For Xerxes and Xtra antisera there was little difference between the titres of each of the bleeds. Xavier antisera displayed lower titres for bleeds 1 & 2 than

A



B



C

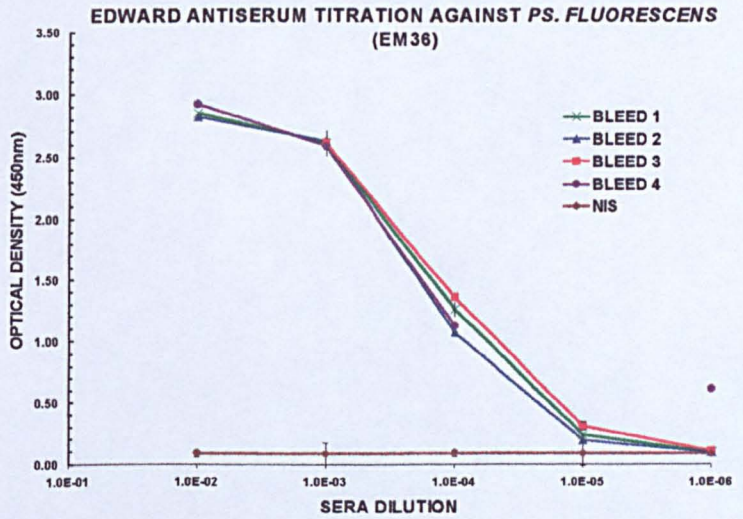
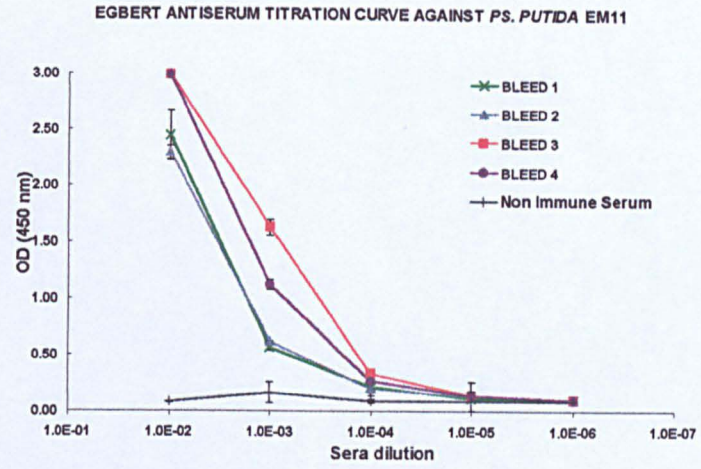


Figure 3.4 Microtitration plate assay of Edward antisera titrated against three of the nine *Ps. fluoresens* isolates used to raise it

In A, B and C the titration was conducted against *Ps. fluorescens* strains EM1, EM28 and EM36 respectively. The sera collected from the rabbit Edward were labelled bleed 1 to 4. Non immune serum (NIS) was used as a control. The error bars at each datum point represent the standard deviation of the duplicate experimental data.

A



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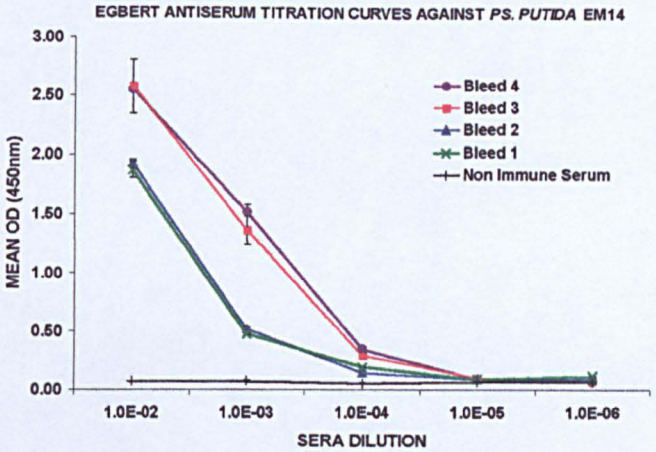


C

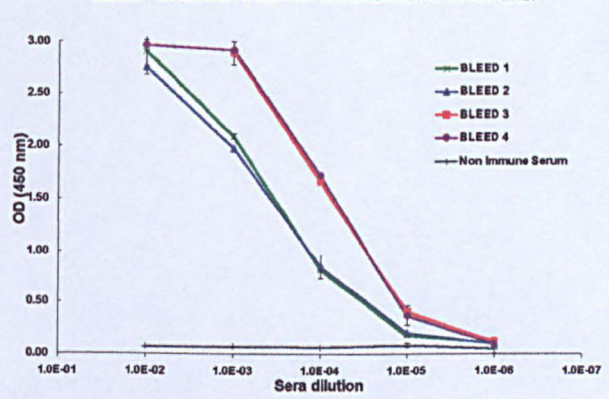
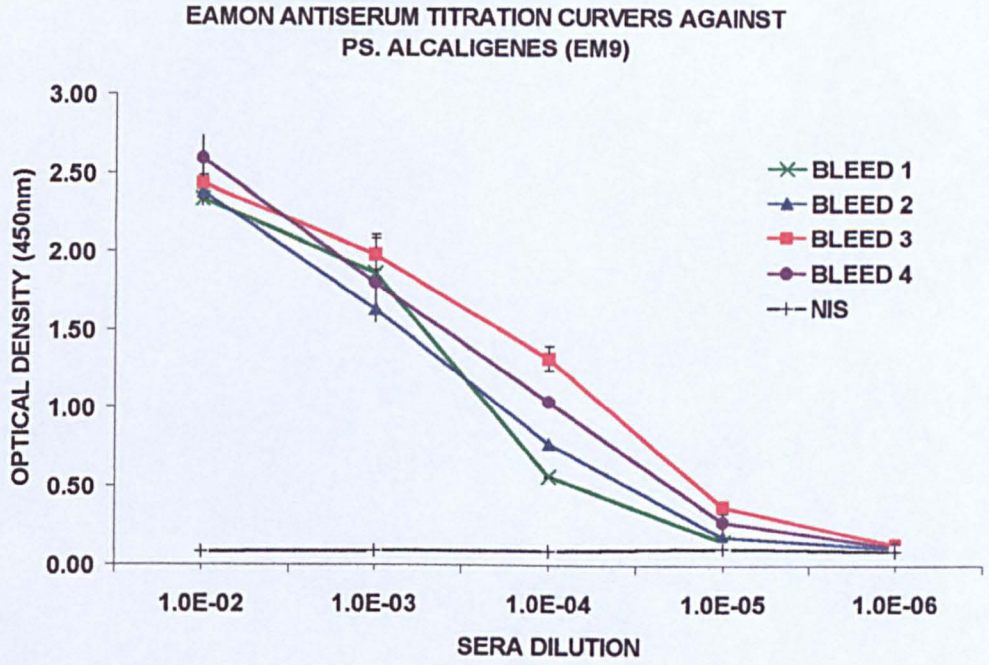


Figure 3.5 Microtitration plate assay of Egbert antisera against three of the five *Ps. putida* isolates used to raise it.

In A, B and C the titration was conducted against *Ps. putida* strains EM11, EM14 and EM20 respectively. The sera collected from the rabbit Egbert were labelled bleed 1 to 4. Non immune serum (NIS) was used as a control. Bleeds 3 and 4 showed a much greater titre of antibodies to the *Ps. putida* cells than bleeds 1 and 2. The error bars at each datum point represent the standard deviation of the duplicate experimental data.

A



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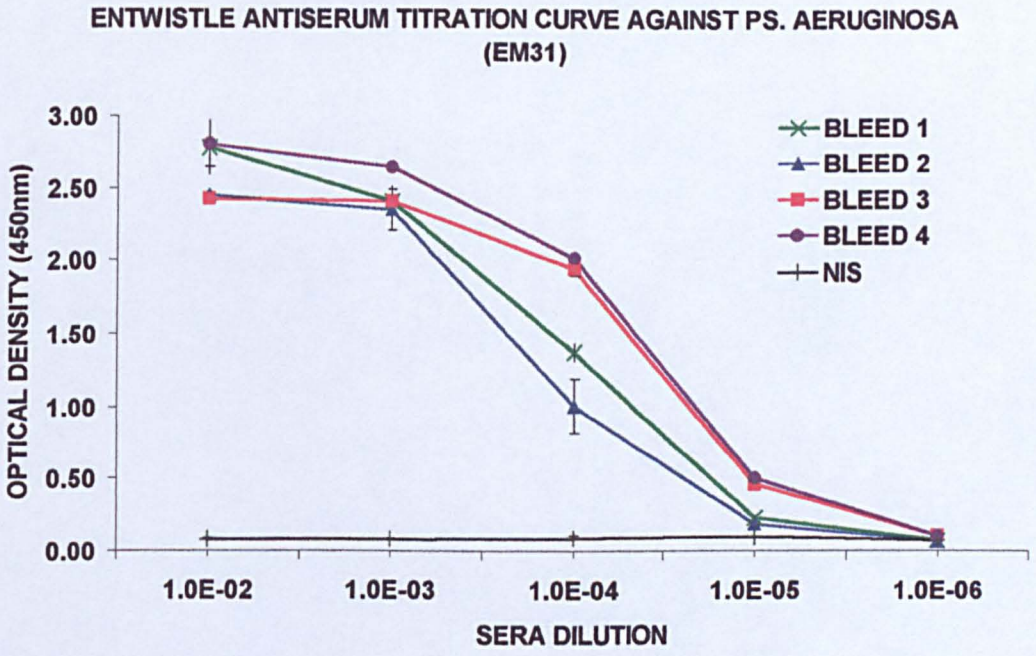
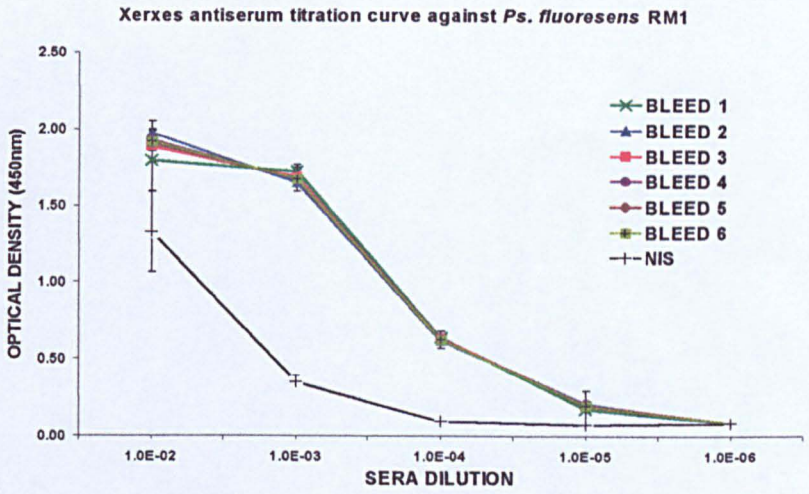


Figure 3.6 Microtitration plate assay of Eamon (A) and Entwistle (B) antisera against each of the isolates used to raise antisera.

The sera collected from each rabbit, Eamon (A) and Entwistle (B) were labelled bleed 1 to 4. Non immune serum (NIS) was used as a control. The error bars at each datum point represent the standard deviation of the duplicate experimental data.

A



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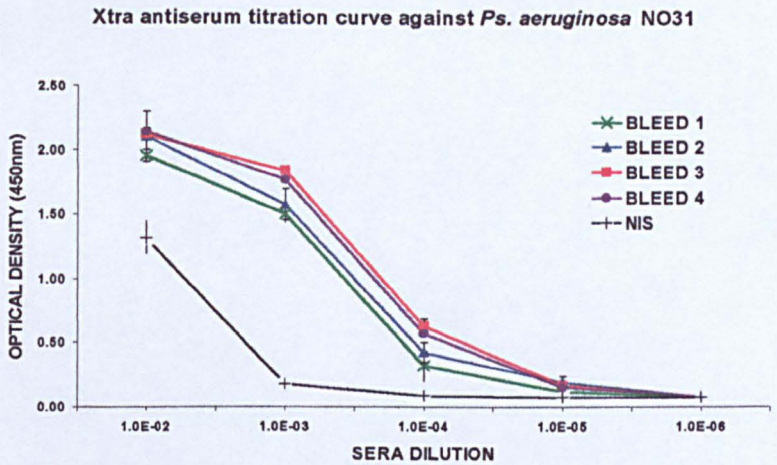

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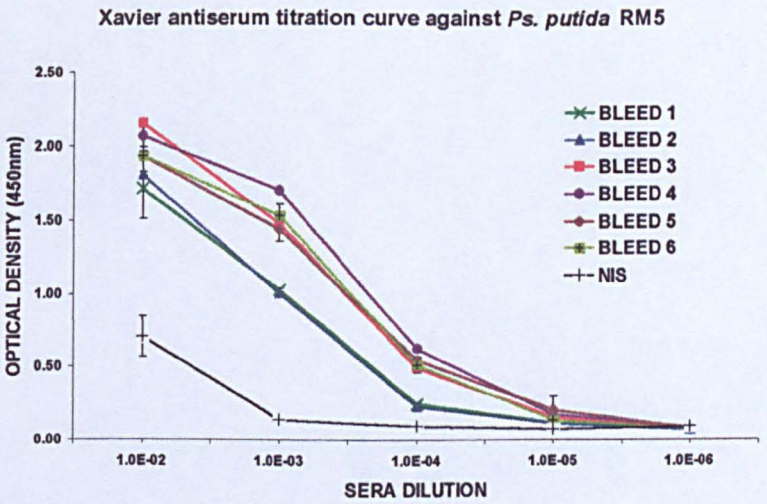
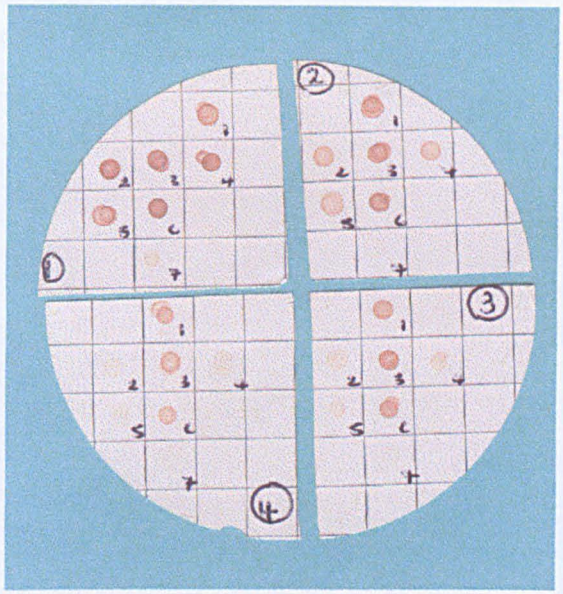


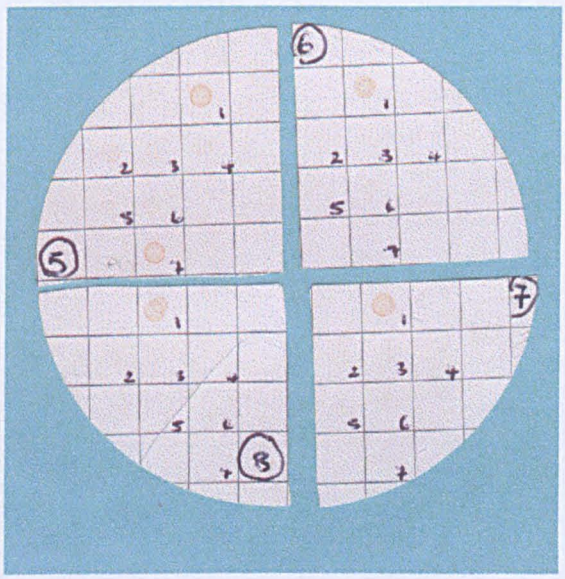
Figure 3.7 The microtitration plate assay of antisera Xerxes (A), Xtra (B) and Xavier (C) against the isolate used to raise them

The sera collected from each rabbit, Xerxes (A), Xtra (B) and Xavier (C) were labelled bleed 1 to 6. Non immune serum (NIS) was used as a control. The error bars at each datum point represent the standard deviation of the duplicate experimental data.



Each filter segment is labelled 1 to 8

1. **Egbert antiserum 10^{-2} dilution**
2. **Egbert antiserum 10^{-3} dilution**
3. **Egbert antiserum 10^{-4} dilution**
4. **Egbert antiserum 10^{-5} dilution**
5. **Non Immune Serum 10^{-2} dilution**
6. **Non Immune Serum 10^{-3} dilution**
7. **Non Immune Serum 10^{-4} dilution**
8. **Non Immune Serum 10^{-5} dilution**

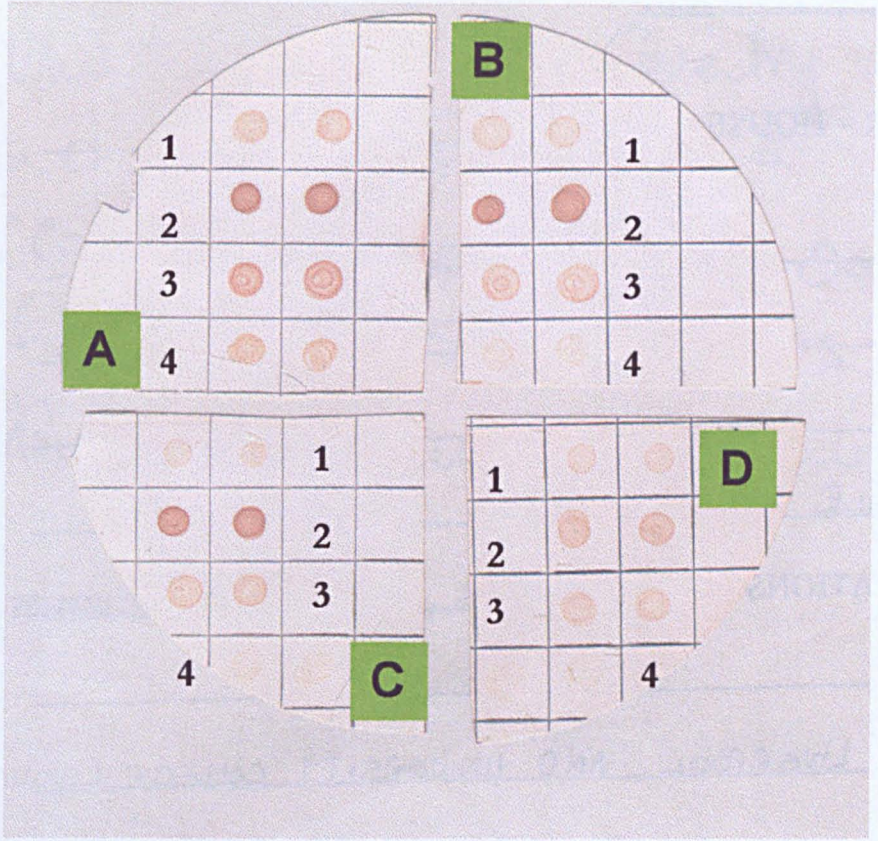


The spots on each filter are numbered 1 to 7 and contain 5µl of standardised cell suspension (0.2 OD_{600}).

1. ***Staphylococcus aureus***
2. ***Ps. putida* EM 11**
3. ***Ps. putida* EM 12**
4. ***Ps. putida* EM 14**
5. ***Ps. putida* EM 17**
6. ***Ps. putida* EM 20**
7. ***Acinetobacter junii***

Figure 3.8 Dot blot titration of Egbert antiserum (bleed 4)

Spots 2-6 represent the isolates used to raise the Egbert antiserum. The Non Immune Serum (NIS) filters were controls. *S. aureus* binds to IgG non-specifically due to the production of protein A. *A. junii* binds to both NIS and Egbert antiserum at high concentrations.



Filter A	Entwistle antiserum 10^{-2}	Cell suspensions spotted in duplicate are numbered 1-4
Filter B	Entwistle antiserum 10^{-3}	1. *Staphylococcus aureus*
Filter C	Entwistle antiserum 10^{-4}	2. *Ps. aeruginosa* EM31
Filter D	NIS 10^{-2} (Control)	3. *Ps. alcaligenes* EM9
		4. *Flavobacterium breve*

Figure 3.9 Dot blot titration of Entwistle antiserum (bleed 4)

The isolate *Ps.aeruginosa* EM31 was used to raise the Entwistle antiserum. At low dilution of the Entwistle antiserum the assay was non-specific and colour developed from cell suspensions of *Ps. alcaligenes.* At 10^{-4} dilution optimum colour was attained for the target organism with little colour development from *S. aureus* and *Ps. alcaligenes.*

bleeds 3, 4, 5 and 6. In general the X series of antisera gave lower antibody titres than the E series.

From the titration curves the dilution that resulted in approximately 60% of antibody binding was used as the working dilution of the sera (Table 3.2). In the case of antisera raised against multiple immunogens the working dilution was calculated from those sera with high antibody titres.

ANTISERA	BLEEDS 1 &2	BLEEDS 3, 4, 5 AND 6
EDWARD	1:10 000	1:10 000
EGBERT	1:5 000	1:50 000
ENTWISTLE	1:10 000	1:50 000
EAMON	1:5 000	1:5 000
XERXES	1:5 000	1:5000
XTRA	1:5 000	1:5 000
XAVIER	1:1000	1:5 000

Table 3.2 Working dilutions for the anti-*Pseudomonas* antisera in microtitration plate assay

3.4.3.2 Dot blot titre

The darkest spots on each filter resulted from the test sera of the lowest dilution (10^{-2}; Fig. 3.8). At a similar dilution of NIS dark spots equal to those seen with the test sera can occur (Fig.3.9). Optimum colour development for each antiserum was defined as being the dilutions that gave strong colour together with little or no colour from the control (all data not shown). The range of dilutions for each antiserum that gave optimum colour is listed in Table 3.3

ANTISERA	DILUTION
Edward	1:100-1:1000
Egbert	1:100-1:1000
Entwistle	1:100-1:1000
Eamon	1:100-1:500
Xerxes	1:100-1:500
Xtra	1:100-1:500
Xavier	1:100-1:500

Table 3.3 Dilutions of antisera that gave optimum colour in the dot blot assay

3.5 DETERMINATION OF CROSS-REACTIVITY

The cross-reactions of the antisera were tested using two methods; non-competitive binding to cells attached to filters and competitive cross-reactions on microtitration plates. Blots facilitated the rapid screening of a large number of strains. The microtitration plate method enabled the examination of cross-reactions in a quantifiable manner.

Antisera were characterised by examining their ability to bind to different species of bacteria. A range of culture collection strains (section 2.4) was tested with each antiserum together with *Pseudomonas* species isolated from spoiled food and the environment.

3.5.1 COMPETITIVE MICROTITRATION PLATE ASSAY

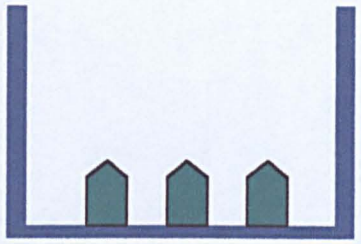
Cross-reactions were tested using a competitive displacement technique as outlined in section 2.5. Figure 3.10 shows a diagrammatic representation of the indirect competitive enzyme linked immuno-sorbent assay (ELISA) approach used. The quantity of antiserum added to the assay is limited so that the number of binding events that can occur will be less than the potential maximum (Wyatt, 1992). The optimum amount of antiserum to be added to the well was determined from a standard curve produced by titrating known amounts of cell suspension against a known quantity of serum with a fixed amount of antigen bound to the well.

Sera raised against multiple immunogens were assayed with either all immunogens coating the plate (1.4 -5μg/ml) or with the immunogens individually coated on to the surface of the microtitration plate (2-3μg/ml). *Ps. fluorescens* NCTC 10038 type strain was coated onto microtitration plates (3-5μg/ml) and assayed in the presence of Edward antisera. Sera raised against a single immunogen were assayed with the immunogen bound to the microtitration plate (3-5μg/ml).

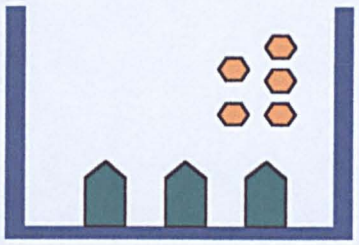
3.5.2 RESULTS

A typical standard curve for a competitive assay (Figure 3.11) indicates the limit of detection (sub-maximal response) for the assay under the stated conditions.

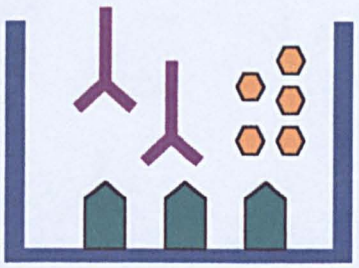
The cross-reactivity of Egbert antiserum with each of the *Ps. putida* strains used to raise it is shown in Table 3.4. At different plate coating levels the cross-reactivity of the assay varies. Microtitration plates coated with EM14 (5μg/ml) cross-reacted with all five of the *Ps. putida* EM strains. At the lower plate coating concentration (1.4μg/ml) displacement was seen with only two of the five strains (EM11 and EM14). When EM12 and EM20 are individually bound to the microtitration plate displacement is seen only with the EM12 and EM 20 respectively. With EM11 and EM17 individually bound to the microtitre plate all the *Ps. putida* EM strains produced displacement.



The microtitration plate wells are coated with a known amount of antigen.

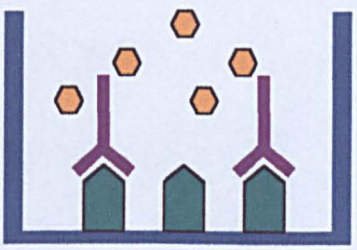


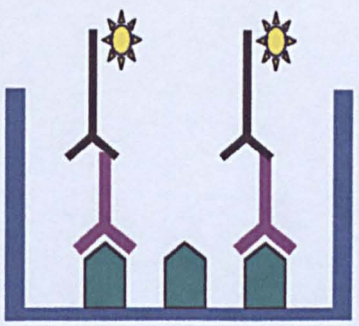
An aliquot of the test sample is added to the well.



A limited amount of antiserum is added to the well and the microtitration plates are incubated.

NEGATIVE OUTCOME

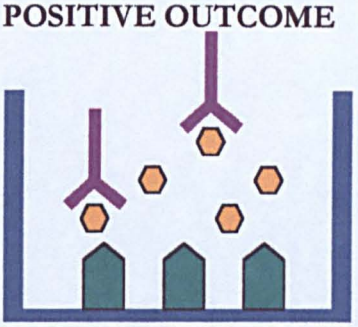




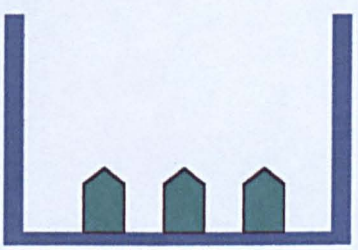
COLOUR

Bound antibody detected with a labelled secondary antibody

POSITIVE OUTCOME



WASH



NO COLOUR

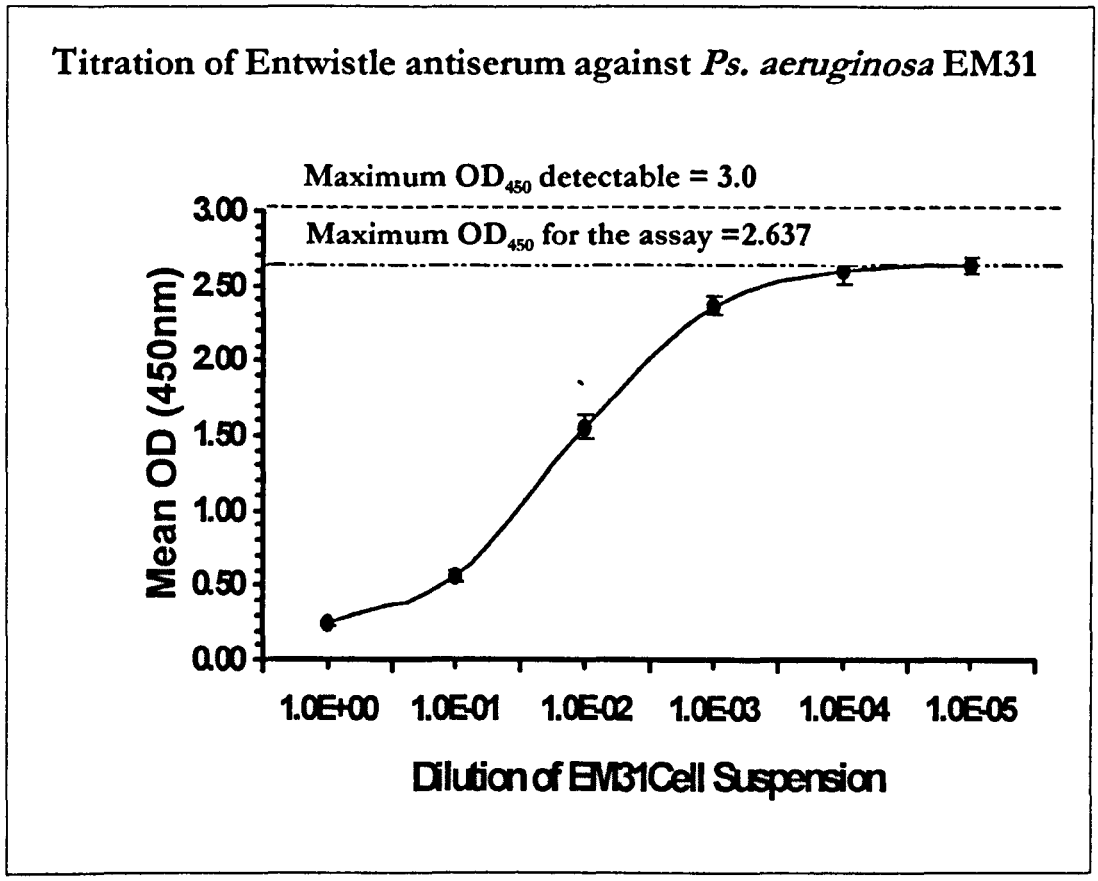
Figure 3.10 Diagrammatic representation of an indirect competitive ELISA

Table 3.5 shows the cross-reactivity of the Edward antiserum. When each of the immunogens that was used to raise the Edward antiserum was individually coated onto the microtitre plate, displacement was invariably seen with the coating organism in suspension; EM1, EM3, EM28, EM33 and EM35 additionally recognised one other EM isolate. The *Ps. fluorescens* type strain was not recognised (data not shown). With all the immunogens for the Edward antisera coated on to the microtitre plate no displacement was seen with any of the coating organisms in suspension.

The competitive assay was conducted with *Ps.fluorescens* NCTC 10038 (type strain) bound to the microtitration plate and Edward antiserum (Table 3.6). Displacement was seen with the type strain in suspension and all but one of the *Ps.fluorescens* EM strains used to raise the Edward antisera (EM29). Cross-reactions were also seen with two *Ps. putida* strains, EM11 and EM14.

Entwistle and Xtra were both raised against *Ps. aeruginosa* and each antiserum only cross-reacted with the *Ps. aeruginosa* isolate in suspension used to raise them (Table 3.7). Entwistle antiserum additionally recognised two *Ps. fluorescens* isolates, EM2 and EM3. Xerxes was raised against a *Ps. fluorescens* isolate (RM1)and displacement was not seen with any of the *Ps. fluorescens* EM isolates used to raise Edward antisera or the type strain. Xerxes antisera cross-reacted with *Ps. putida* isolate (RM5) that was used to raise the Xavier antibody. Eamon antisera recognised the organism used to raise it in suspension and *Ps. putida* EM11.

The pattern of cross-reactions seen with the Xerxes and Xavier antisera against *Pseudomonas* isolates from milk were identical (Table 3.7A). The pattern of cross-reactivity seen with *Pseudomonas* isolates from soil and water were also very similar (data not shown).



Dilution of EM31 cell suspension	[Protein] (μg/ml)	Mean OD 450nm	SD (+/-)
1.0E-05	0.008	2.637	0.052
1.0E-04	0.083	2.604	0.095
1.0E-03	0.830	2.361	0.059
1.0E-02	8.300	1.559	0.085
1.0E-01	83.000	0.552	0.035
1.0E+00	833.000	0.247	0.017

Figure 3.11 Standard curve for a limited-reagent competitive assay for Entwistle antiserum ($5x10^{-5}$) against *Ps. aeruginosa* EM31 with EM31 bound to the microtitration plate (5μg/ml)

The maximum OD_{450} detectable is 3.0. The maximum OD_{450} for the standard curve is ~2.6. Therefore, the amount of Entwistle antiserum used (50μl of 5x 10^{-5} dilution) is limiting when 5μg/ml of antigen is bound to the plate. In a limited reagent competitive assay if all the antibodies in suspension were to bind to the bound antigen coating the plate then the OD_{450} subsequently attained should be below the maximum OD_{450} detectable of 3.0. The detection limit for the assay, which from the table of results, can be calculated to be approximately 100ng/ml of EM31 protein.

Each datum point represents the mean of duplicate determinations and the standard deviations.

STRAINS COATED ON PLATE	CROSS-REACTIONS	
	5μg/ml serum coating plate	1.4μg/ml serum coating plate
EM11, EM12, EM14, EM17 & EM20	EM12 & EM20	EM12 & EM20
EM11	EM11, EM12, EM14, EM17 & EM20	EM11, EM12, EM14, EM17 & EM20
EM12	EM12,	EM12,
EM14	EM11, EM12, EM14, EM17 & EM20	EM11 & EM14
EM17	EM11, EM12, EM14, EM17 & EM20	EM11, EM12, EM14, EM17 & EM20
EM20	EM20	EM20

Table 3. 4 Competitive assay cross-reactions of the *Ps. putida* strains used to raise the Egbert antiserum

STRAINS COATED ON PLATE	CROSS-REACTIONS
EM1	EM1 & EM33
EM2	EM2
EM3	EM3 & EM28
EM27	EM27
EM28	EM3 & EM28
EM29	EM29
EM33	EM1 & EM33
EM35	EM35 & EM36
EM36	EM36
ALL	NO DISPLACEMENT SEEN

Table 3.5 Competitive assay cross-reactions of the *Ps. fluorescens* strains used to raise the Edward antiserum.

TEST STRAINS	CROSS-REACTIONS
Ps. fluorescens NCTC 10038	+
EM1	+
EM2	+
EM3	+
EM27	+
EM28	+
EM29	-
EM33	+
EM35	+
EM36	+
Ps. putida EM11	+
EM12	-
EM14	+
EM17	-
EM20	-
Ps. alcaligenes EM9	-
Ps. aeruginosa EM31	-
Ps. aeruginosa NCIMB 10545	-
Ps. alcaligenes NCTC 10367	-

Table 3.6 Competitive assay cross-reactions for Edward antiserum ($5x10^{-4}$) with *Ps.fluorescens* NCTC 10038 (4μg/ml) bound to plate.

TEST SAMPLE	CROSS REACTIONS				
	Eamon EM9 bound	Entwistle EM31 bound	Xerxes RM1 bound	Xavier RM5 bound	Xtra NO31 bound
Staphylococcus aureus	-	-	-	-	-
Ps. aeruginosa (type strain)	-	-	-	-	-
Flavobacterium breve	-	-	-	-	-
Acinetobacter junii	-	-	-	-	-
Ps. fluorescens (type strain)	-	-	-	-	-
Alcaligenes faecalis	-	-	-	-	-
Ps. fluorescens EM1	-	-	-	-	-
Ps. fluorescens EM2	-	+	-	-	-
Ps. fluorescens EM3	-	+	-	-	-
Ps. fluorescens EM27	-	-	-	-	-
Ps. fluorescens EM28	-	-	-	-	-
Ps. fluorescens EM29	-	-	-	-	-
Ps. fluorescens EM33	-	-	-	-	-
Ps. fluorescens EM35	-	-	-	-	-
Ps. fluorescens EM36	-	-	-	-	-
Ps. putida EM11	+	-	-	-	-
Ps. putida EM12	-	-	-	-	-
Ps. putida EM14	-	-	-	-	-
Ps. putida EM17	-	-	-	-	-
Ps. putida EM20	-	-	-	-	-
Ps. aeruginosa EM31	-	+	-	-	-
Ps. alcaligenes EM9	+	-	-	-	-
Ps.fluorescens RM1	-	-	+	+	-
Ps.putida RM5	-	-	+	+	-
Ps. aeruginosa NO31	-	-	-	-	+

Table 3.7 Competitive assay cross-reactions for Eamon, Entwistle, Xerxes, Xavier and Xtra antisera

TEST SAMPLE (milk isolates)	CROSS REACTIONS	
	Xerxes RM1 bound	Xavier RM5 bound
RM1	+	+
RM2	+	+
RM3	+	+
RM4	+	+
RM5	+	+
RM6	+	+
RM7	+	+
RM8	+	+
RM13	+	+
RM14	-	-
RM19	-	-
RM20	-	-
RM23	+	+
RM24	-	-
M1	-	-
M2	-	-
M3	-	-
M4	-	-
M5	-	-
M6	-	-
M7	-	-
M8	-	-

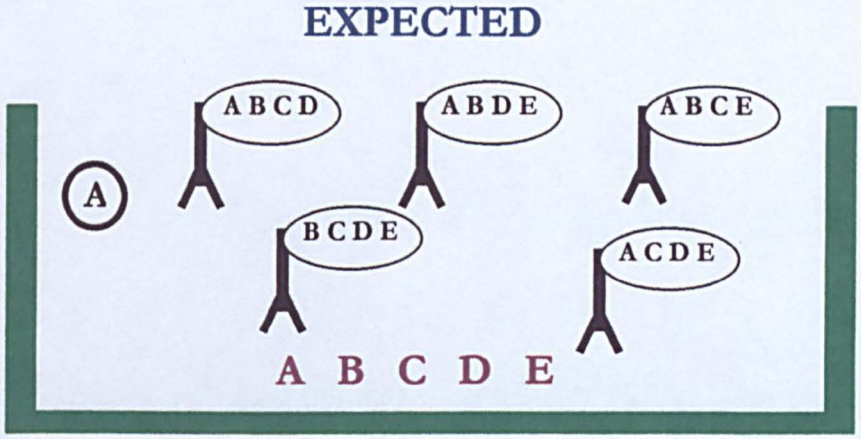
RM = RAW MILK M= PASTEURISED MILK

Table 3.7 A Competitive assay cross-reactions for Xerxes and Xavier

3.5.3 DISCUSSION

The outcome of the competitive ELISA for Edward and Egbert antisera was directly related to which of the bacterial immunogens was bound to the microtitre plate. The selective specificity of the competitive assay, for sera raised against multiple bacterial immunogens, is a phenomenon previously unreported (Johnson *et al.*, 1996). The expectation was that each of the multiple immunogens used to raise antisera would have common epitopes such that the polyclonal antibody would recognise immunising strains in suspension, regardless of which particular one was bound to the plate.

Egbert antisera was derived from a rabbit immunised with five strains (labelled A,B,C,D and E for illustration purposes) and would be expected to have many shared antigens and the polyclonal antibody would recognise all strains more or less equally (illustrated below).



With all the immunising strains coating the microtitre plate, 80% of the polyclonal pool will recognise immunogen A in suspension leaving only 20% binding to the plate. Displacement would be easily recognised for each immunogen in suspension.

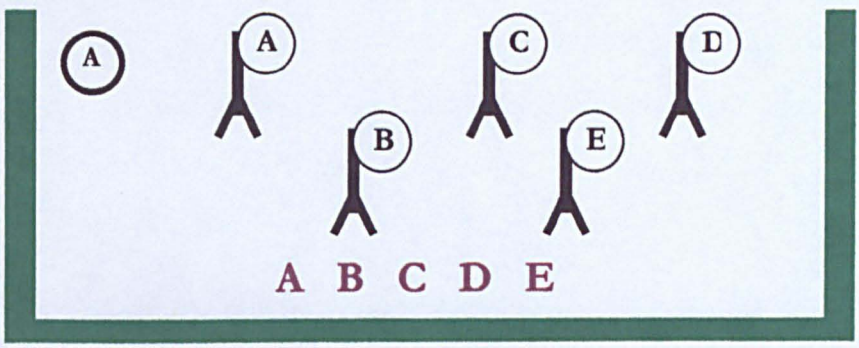
Of course, in reality the distribution or affinity of shared antigens may not be equal. All of the Egbert immunogens in suspension did not cause displacement when the microtitre plate was coated with all immunogens. EM12 and EM20 were recognised and EM11, 14 and 17 were not. There may be a larger population of EM12 and EM20 antibodies in the polyclonal pool than EM11, 14 and 17 or additional mechanisms at work.

Edward antisera were raised against nine bacterial immunogens. In the situation when all the immunogens were bound to the plate none of the immunogens in suspension cross-

reacted. Non recognition could be due to conformational changes occurring during the binding of the cells to the microtitration plate. Epitopes on the bound cell surface may be altered or made available, which are more highly specific for the antiserum than the epitopes on the unbound immunogen cell suspension.

An alternative theory to describe the observed results for Edward antisera is that the immune system of the rabbit recognised each immunising isolate as distinct antigens such that each immunogen had no common or cross-reacting epitopes (shown below). In this scenario only 11% of the polyclonal pool would recognise antigen A in suspension leaving 89% bound to the microtitre plate and displacement would be difficult to recognise.

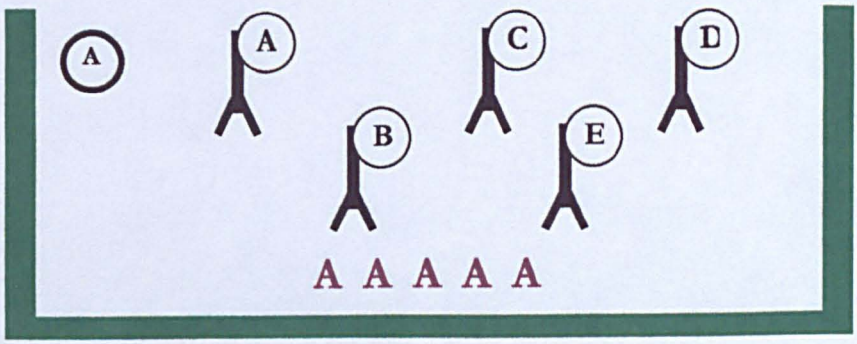
ALL STRAINS COATING THE WELLS



If the 'distinct antigen' theory is expounded further to incorporate the results for Edward antiserum with single immunogen coated wells, then the theory falls down. With one immunogen bound to the plate (as illustration i below) 11% of the antibody pool can bind to the plate (a proportion should bind to A in suspension) and all of the remainder (89%) can be displaced. Displacement of A in suspension with A bound, would be easily recognised (as seen with Edward antisera).

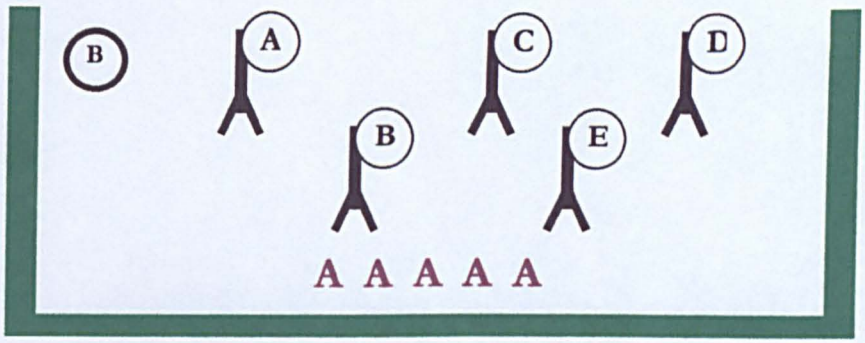
DISTINCT ANTIGENS

ONE STRAIN COATING THE WELLS (i)



DISTINCT ANTIGENS

ONE STRAIN COATING THE WELLS (ii)



With immunogen B in suspension and A bound to the plate (illustration ii), 11% of the polyclonal pool can bind to the plate and the remainder should be displaced (89%). Hence, displacement of B in suspension with A bound to the plate would be easily recognised. With respect to Edward antiserum, the displacement of the equivalent of immunogen B in suspension with immunogen A bound to the plate does not occur. The data attained suggest that all of the antibodies binds to the bound immunogen when B was in suspension.

The mechanisms of selective specificity seen with Edward antiserum cannot be fully explained but could have useful applications. By varying the immunogen bound to the plate the screening for a single specific target can be attained with a single antibody population generated from multiple immunogens. Manipulating the polyclonal antibody in such a way could vastly increase its utility.

Coating the microtitre plate with *Ps. fluorescens* NCTC 10038 (type strain) and conducting the competitive assay with Edward antisera the assay recognises all *Ps. fluorescens* isolates (except EM29). With the type strain bound to the microtitre plate the range of organisms previously detected widened and reflected the pattern of cross-reactivity found with the non-competitive assay.

Xerxes and Xavier antisera were raised against different species of *Pseudomonas* (*Ps.fluorescens* and *Ps.putida,* respectively) and yet the pattern of cross-reactivity that they produced were identical. The only common parameter that the two antisera share was that they were both isolated from raw milk. The cross-reactivities of the Xerxes and Xavier antisera were very

niche specific as they were able to distinguish between *Pseudomonas* isolates from raw milk and those from other sources, such as soil or pasteurised milk.

3.5.4 DOT BLOT

Colony blots were used to assess the proportion of naturally occurring pseudomonads that could be stained using the chosen antisera. Seventy-five isolates (Table 5.2) with typical *Pseudomonas* morphology were selected from CFC agar. Each colony was transferred directly onto nitrocellulose filters with a sterile toothpick. The procedure was duplicated using standardised cell suspensions (0.2 OD_{600}) and stained as outlined in section 2.4. The ability of the working dilution of each antiserum to bind to each isolate was appraised by visually comparing the colour of each colony spot stained with the non-immune serum control.

3.5.5 RESULTS AND DISCUSSION

ANTISERUM	PERCENTAGE CROSS REACTION
Egbert	46
Edward	40
Entwistle	37
Eamon	54
Xerxes	63
Xavier	61
Xtra	44

Table 3.8 Dot-blot percentage cross-reactions of each antiserum with naturally occurring *Pseudomonas* isolates

EM STRAIN CODES	EDWARD		EGBERT	
	Dot-blot	ELISA EM1 BOUND	Dot-Blot	ELISA EM20 BOUND
Ps fluorescens 36	+	-	-	-
Ps fluorescens 35	(+)	-	-	-
Ps fluorescens 33	+	+	-	-
Ps fluorescens 29	+	-	-	-
Ps fluorescens 28	(+)	-	-	-
Ps fluorescens 27	+	-	-	-
Ps fluorescens 3	+	-	-	-
Ps fluorescens 2	+	-	-	-
Ps fluorescens 1	+	+	-	-
Ps. putida 20	-	-	+	+
Ps. putida 17	-	-	-	-
Ps. putida 14	+	+	-	-
Ps. putida 12	-	-	-	-
Ps. putida 11	+	-	+	-

( ) = Borderline reaction

Table 3.9 Dot blot and Competitive ELISA cross-reactivity of Edward and Egbert antisera

All borderline results were recorded as positive. All the isolates that were positive in the competitive ELISA format were positive in the dot blot format. However, the non-competitive assay produced more positive results than the competitive assay (example shown in Table 3.9). Virtually no binding was seen on the non-immune serum blot with the exception of *S. aureus*.

Staining of colonies that were transferred onto membranes with a toothpick produced visually more clear-cut results than the cell suspensions. The ease of assessing each cross-reaction by eye must be contextualised due to the lack of standardisation between successive samples. Non-specific cross-reactions may occur due to high cell numbers.

The reaction patterns for Egbert, Edward, Entwistle and Xtra antisera were quite different indicating that each sera cross-reacts with a different sub-population (with some overlap). The cross-reactivity of Xerxes and Xavier (both antisera were raised against milk isolates of different species) was near identical. Xavier and Edward antisera were raised against *Ps. fluorescens* and Xerxes and Egbert were raised against *Ps. putida* however, both pairs of antisera defined distinct sub-populations with some overlap. Xerxes and Xavier antisera cross-reacted with the most environmental isolates (63% and 61% respectively) and Entwistle cross-reacted the least (37%; Table 3.8). Eamon antisera produced a large proportion of borderline cross-reactions, which were scored positive. Entwistle antiserum (raised against *Ps. aeruginosa*) defined a cohesive sub-population with little overlap between isolates recognised by other antisera.

The failure to recognise all members of each *Pseudomonas* species is not significant if a marker organism is to be followed within a model system but should be considered within natural systems. For locational studies the use of probes that recognise a sub-population is justified as long as that population can be defined.

Pseudomonas species are known to carry several plasmids that may confer various phenotypic traits (Bergey's Manual, 1984). Each plasmid can be readily lost from environmental isolates on sub-culturing and that may affect bacterial physiology. Over time variable reactions were seen with two or three environmental isolates from the soil, notably, in the non-competitive assay.

3.6 LIMITATIONS OF THE ANTISERA

From the cross-reactions it would appear that most of the produced antisera, whether raised to a single isolate or nine isolates, were specific for a subset of *Pseudomonas* broadly along species lines and within that subset there were a wide range of staining reactions. At the working concentration of each antiserum no cross-reactions were seen outside the genus. However, the variation in staining reactions could limit the use of the antisera to enumerate all environmentally isolated bacteria or to prove definitively that an unknown bacterium is not a *Pseudomonas*.

Antibody-linked staining can be mediated with these antibodies confidently for the identification of *Pseudomonas* in sections or to locate sites capable of supporting *Pseudomonas* growth. It must be born in mind that absence of stained cells does not mean that *Pseudomonas* cannot be present.

3.7 EVALUATION OF ANTIBODY BINDING

The aim of these investigations was to determine where the antibodies were binding on the surface of the bacterial cell. *Pseudomonas* are known to produce exopolysaccharide which can be highly immunogenic. If binding only occurred with the exopolysaccharide then adequate whole cell staining may not occur in all circumstances. During the production of the polyclonal antibodies the bacterial immunogens were washed under low stringency conditions so that the exopolysaccharide was not removed. However, if antibody were binding to the lipopolysaccharide (LPS), which form an intergral part of the outer membrane, or protein molecules then whole cell staining under most conditions may be assured.

Cells were antibody-linked stained and visually examined using Atomic Force Microscopy and Scanning Electron Microscopy.

3.7.1 TOTAL CELL PROTEIN AND LPS

The method for extracting, running and immunoblotting the total cell protein and LPS are outlined in sections 2.15 -2.17.

3.7.1.1 Results and discussion

The Edward antibody binds to protein and LPS. Figure 3.12A shows the total cell protein of *Ps. fluorescens* EM strains run on a protein gel and stained with Coomassie blue to visualise the bands. Each sample exhibited similar multiple band patterns from the total cell proteins that originated from inside as well as outside the cell. Figure 3.12B shows the total cell protein immuno-blot. Multiple bands are recognised by the Edward antisera over a wide range of sizes.

Figure 3.13A shows the LPS gel silver stained to visualise the bands. The patterns generated by the immuno-blots (Fig 3.13B & C) were ladder-like and that of EM1 was longer than that of EM31. The rungs of the ladder related to the repeated sugar sequences in the LPS.

3.7.2 Atomic Force Microscopy

3.7.2.1 Introduction

The combination of Atomic Force Microscopy (AFM) with electrophysiological techniques allows the imaging of surfaces of living cells under physiological conditions at sufficient resolution to detect processes such as antibody-antigen interactions over a period of time (Gunning *et al.*, 1996).

AFM spans the resolutions achieved for light microscopy and electron microscopy without the limitation of extensive sample preparation. The AFM is a probe microscope and looks more like a record player than a microscope (Picture 3.1). The probe consists of a flexible cantilever with a silicon nitride pyramid tip (3μm deep) and measures the sample surface topography up to 3μm deep. A low power laser light is aimed at the cantilever and reflected onto a photodiode detector. As the cantilever bends, due to fluctuations in sample surface, the reflected laser light moves across the photodiode detector that registers the movement. Mirrors are placed between the cantilever and the diode to exaggerate this movement which allows sub-nanometer changes on the sample surface to be detected (Figure 3-14).

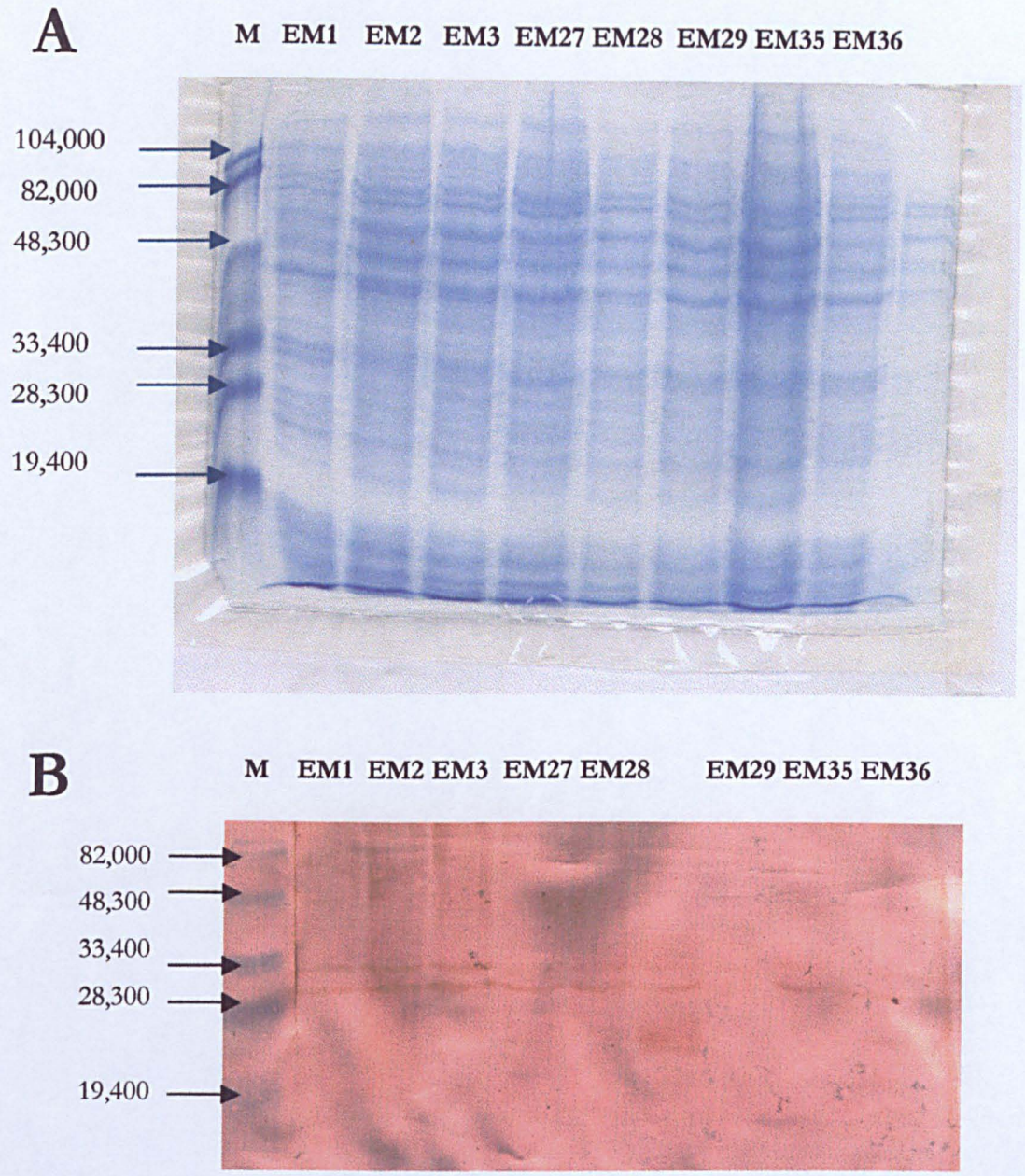


Figure 3.12 Total cell protein from Ps. fluorescens EM strains (12% gel)

One gel was stained with Coomassie blue (A) and the protein bands from the other gel were transferred onto a nitrocellulose membrane and immuno-blotted with Edward antisera ($5x10^{-3}$)

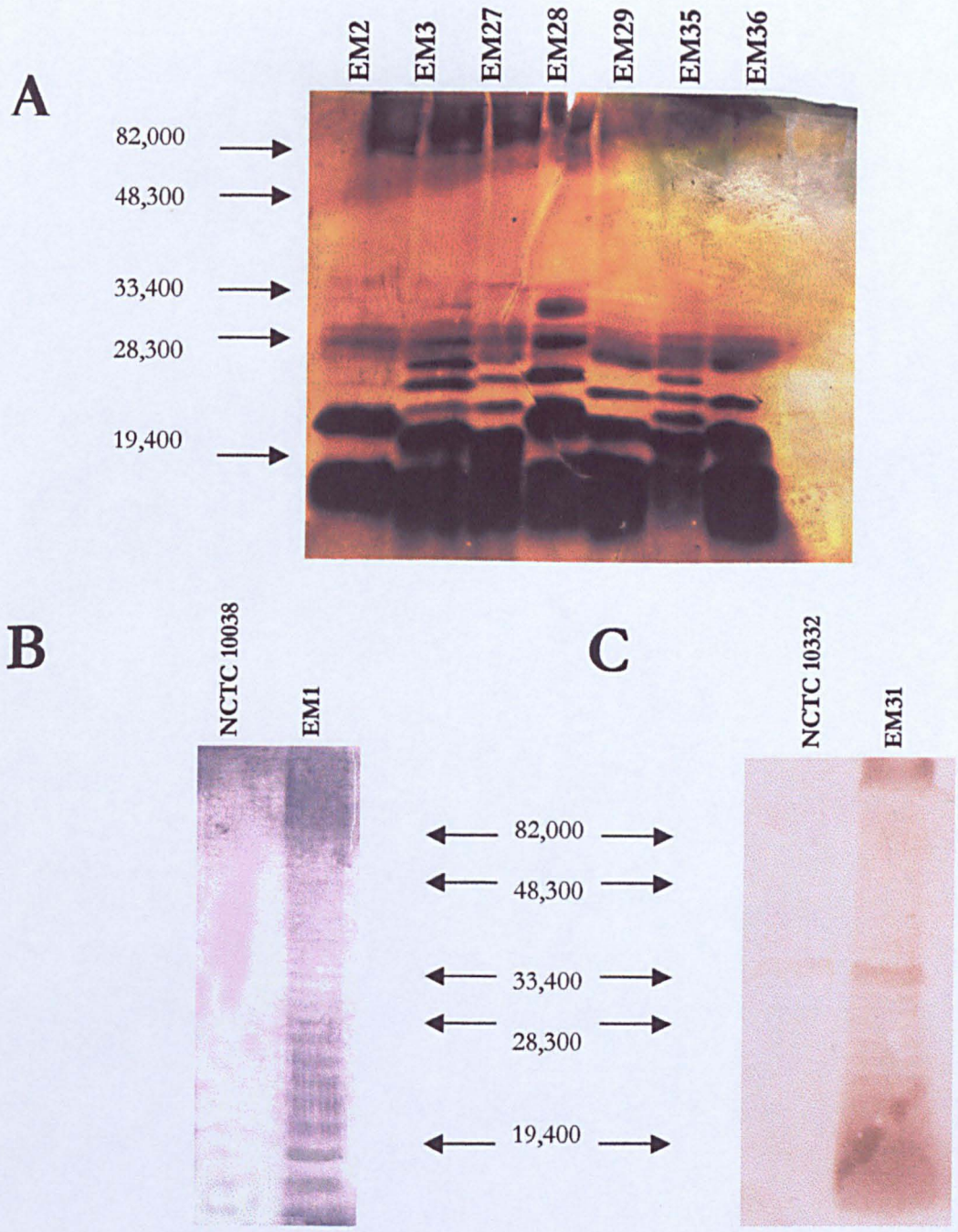


Figure 3.13 Total LPS of *Pseudomonas* strains silver stained (A) and immuno-blotted (B & C)

Ps. fluorescens lipopolysaccharide was run on a 15% gel and silver stained (A). *Ps. flurorecens* EM1 and type strain LPS were immuno-blotted in the presence of Edward antiserum ($5x10^{-4}$; B). No cross-reactivity was seen with the type strain (NCTC 10038) and a ladder pattern was seen for the EM1 strain representing the sub-units of the long chain fatty acids. *Ps. aeruginosa* NCTC 10032, type strain and EM31 LPS were immuno-blotted in the presence of Entwistle antiserum ($1x10^{5}$) and a typical ladder pattern was seen for the EM31 strain (C). A predominant antigenic band of approximately 35KDa was detected in both the EM31 strain and the *Ps. aeruginosa* type strain.

Samples for examination by AFM are usually studied in a liquid cell into which all components (sample, stylus and laser optics) are immersed. The liquid cell accommodates the imaging of biological samples in their native form (Kirby and Morris, 1994). The major limitation to this system is the degree of roughness of the surface which should be small compared to the overall sample size. Large objects such as bacterial cells can be viewed on glass cover slips but smaller objects such as proteins are placed onto atomically flat mica.

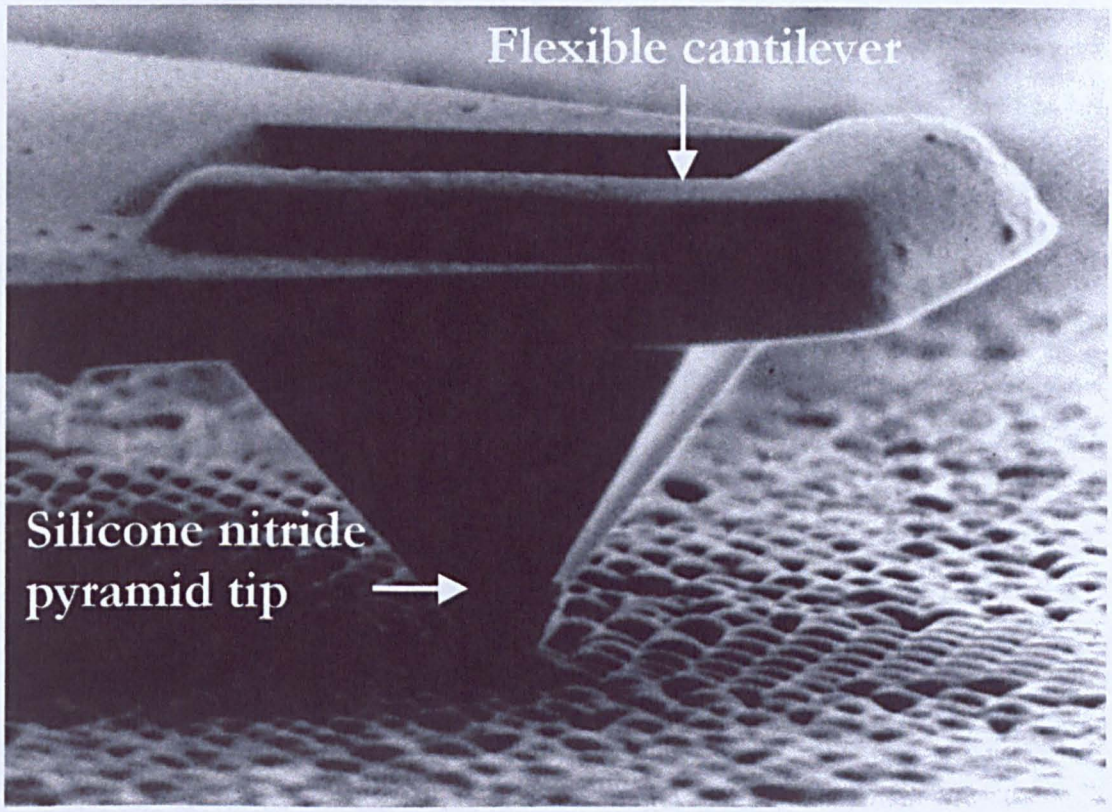
The aim of this investigation was to see if the AFM could be used to locate the antibody-antigen reaction within a *Pseudomonas* biofilm.

3.7.2.2 Sample preparation

Pseudomonas fluorescens (RM1 and RM5) and *Pseudomonas aeruginosa* (NO31) were grown up statically (24-48h at 25°C) in brain heart infusion broth (10 ml). Aliquots (10 ml) of MOPS (a chemically defined medium containing minimal surface-active molecules that aid the formation of a clean interface) were inoculated with each culture (10^3 cfu/ml). Each aliquot of MOPS was poured aseptically into a pre-sterilised flat-bottomed glass dish and covered with a layer of hexadecane. The bacteria were incubated statically for 2 h at 25°C to allow the formation of a bacterial film at the hexadecane-water interface (Gunning *et al.*, 1996). Freshly cleaved 1cm^2 of mica was lowered into aqueous culture medium under the oil layer and drawn up through the biofilm interface attaching a portion of the single culture biofilm to the mica surface. The mica pieces were then rinsed in pentane to remove the hexadecane. A visual check was made to see if attachment had taken place using a light microscope. The samples were then allowed to air dry.

3.7.2.3 Antibody staining

The pure culture monolayers attached to the mica were incubated with the appropriate primary antibody for 20 min at 37°C and then washed four times in PBST. A secondary antibody, anti-rabbit IgG labelled with 15nm diameter gold spheres (Sigma) was added, incubated (20 min at 37°C) and washed four times in PBST. A silver enhancer (Biocell) was added (20 min at 37°C) before examination by AFM.



Picture 3.1 Photograph of the atomic force microscope probe

(Photograph courtesy of P.A. Gunning, Institute of Food Research, Norwich)

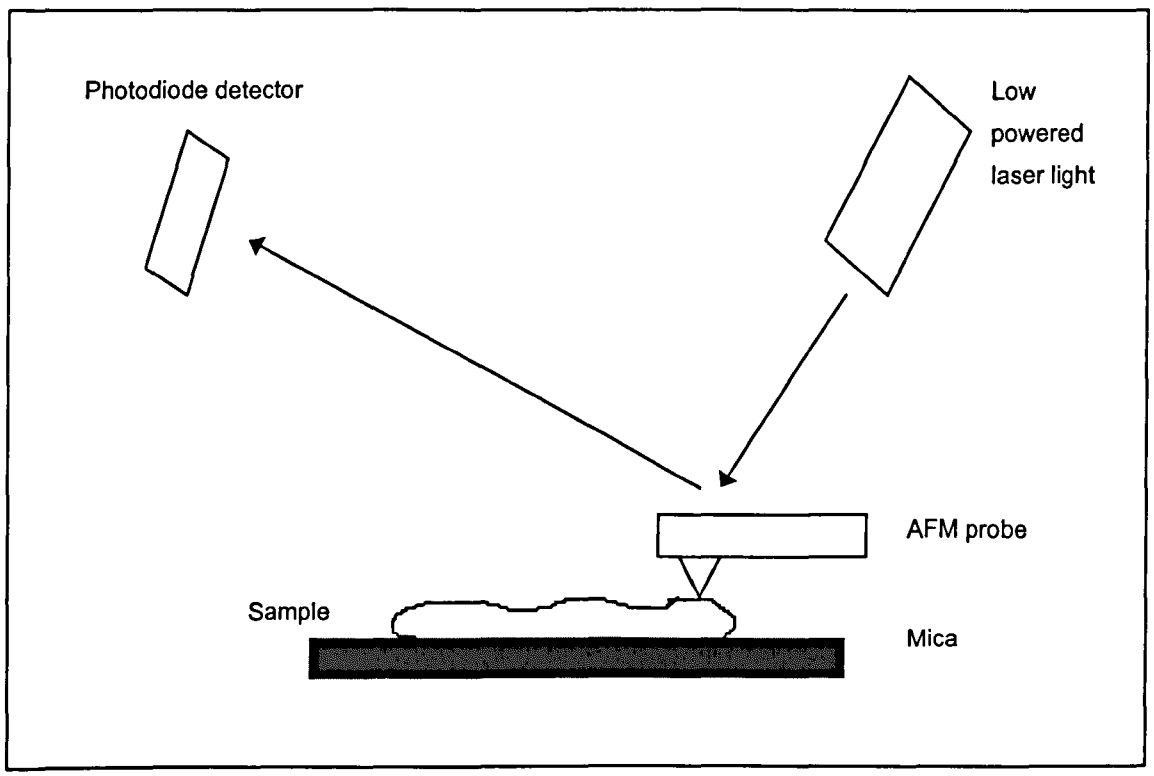


Figure 3.14 Diagrammatic representation showing how the Atomic Force Microscope works.

A low powered laser light is aimed at the AFM probe and reflected onto a sensitive photodiode detector. As the cantilever bends due to fluctuations on the sample surface, the reflected laser light moves across the photodiode detector registering the movement.

3.7.2.4 Atomic Force Microscope

Samples were examined using an East Coast Scientific (ECS) Ltd (Cambridge, UK) AFM. The operating mode employed was DC contact. This is the simplest way of generating an image with a constant force being applied to the probe.

3.7.2.5 Results and discussion

On first conducting this experiment the *Pseudomonas* biofilm failed to remain attached to the mica throughout the antibody staining procedure. This problem recurred on several occasions. Attachment was established on some pieces of mica on a variable basis and these pieces were viewed using AFM.

Viewing of the *Pseudomonas* biofilms using AFM proved difficult. The resolution of the images of the biofilm produced by the AFM was very poor. In theory the AFM can resolve an image to molecular or atomic resolution. However the resolution achievable diminishes as the surface roughness increases. The surface becomes rougher due in part to the production of exopolysaccharide and/or the cell distribution within the biofilm being uneven (Gunning *et al.*, 1996). If differences in the topographical heights within the sample are greater or of the same size as the tip of the AFM (3μm) then the cantilever will lift the tip from the surface and prevent imaging. No AFM images were produced of the three biofilms made. AFM images of *Ps.putida* biofilm have been attained by Gunning *et al.* (1996) using a similar technique.

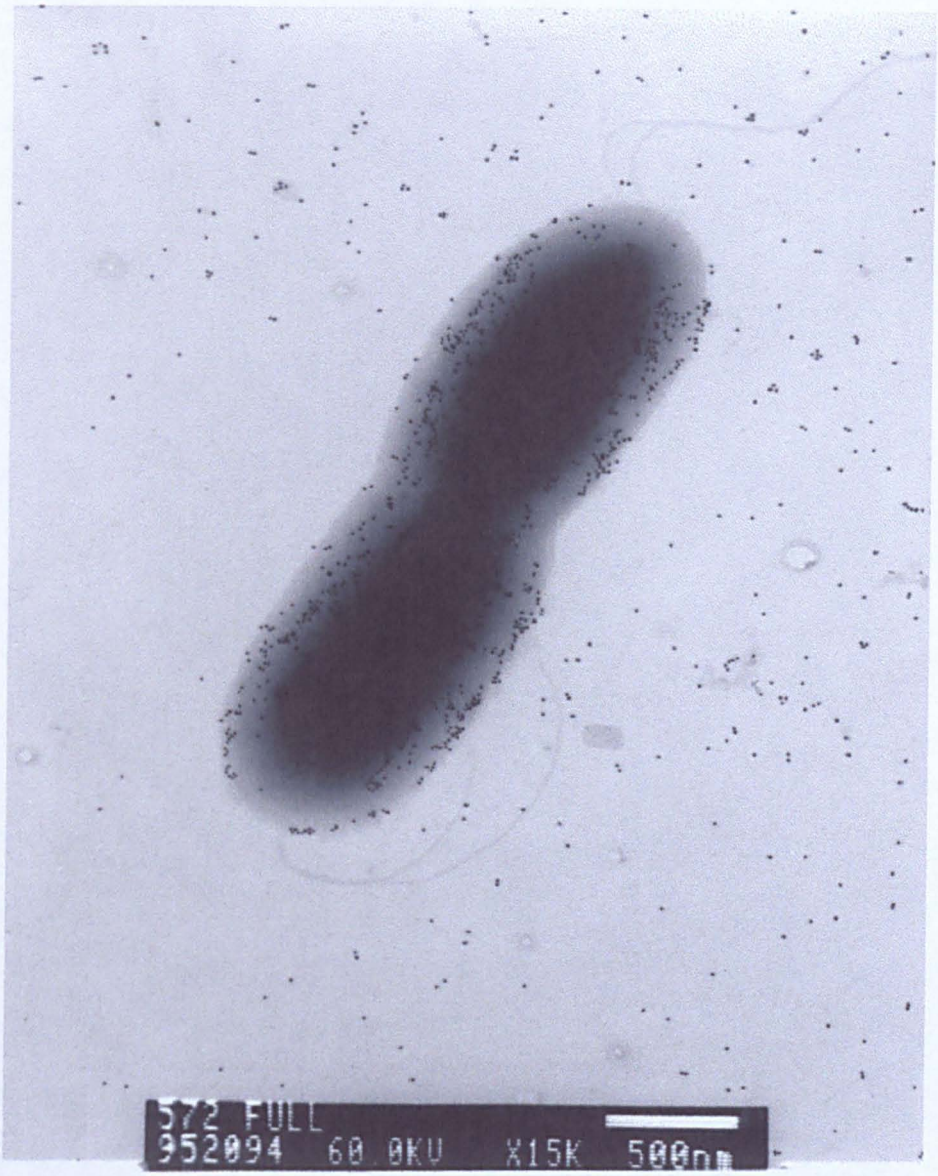
The main objective of these experiments was to use the AFM together with antibody -linked staining to visualise where on the cell surface the antibody was binding. Due to numerous technical problems this objective was unattainable. Further work was necessary to optimise the AFM parameters (using glass surface, changing tip force or using a different type of cell) so that imaging of these biofilms could be attained. However, the devotion of the time and effort necessary to achieve this goal would be beyond the scope of this project.

3.7.3 SCANNING ELECTRON MICROSCOPY

Dr Mary Parker (Institute of Food Research, Norwich) kindly conducted the antibody-linked staining of the *Ps. putida* EM14 cells and produced scanning electron micrographs (using a Leica Cambridge Steroscan 360 scanning electron microscope). The secondary antibody used to detect the primary antibody was labelled with 15nm gold spheres, which are electron dense to assist visualisation under the SEM, and appear as black dots.

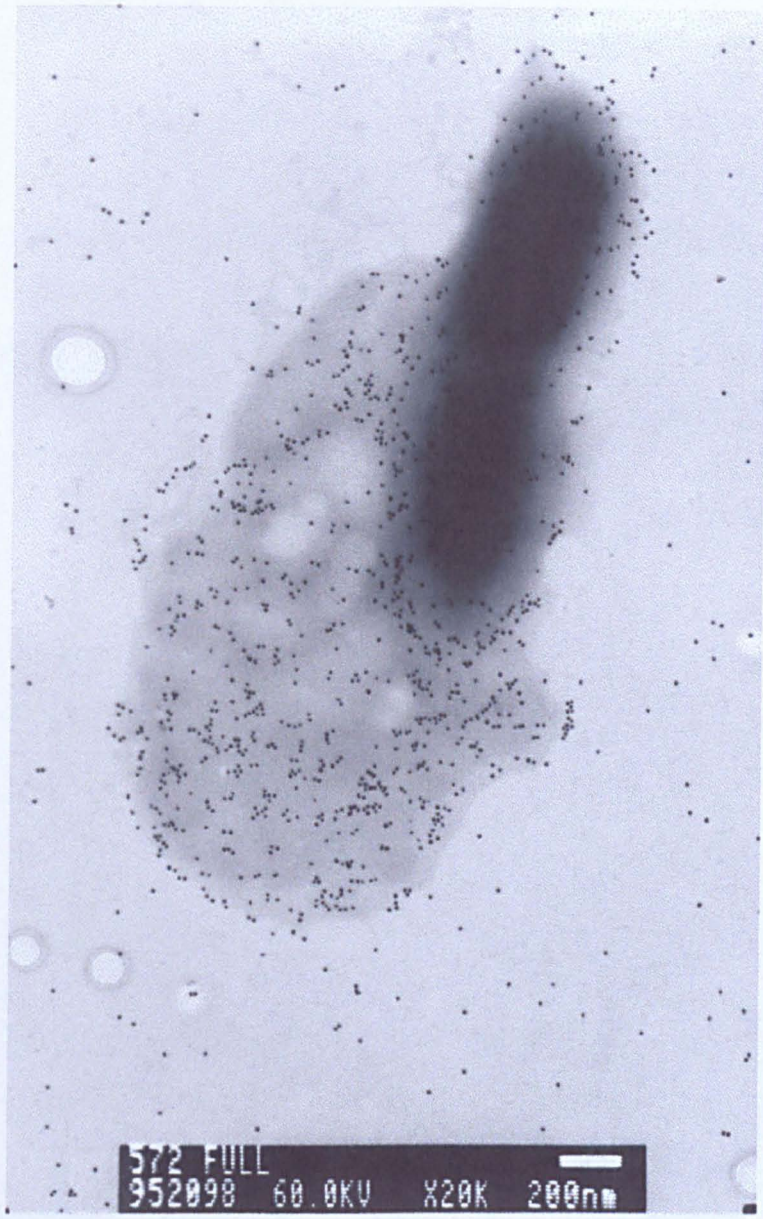
3.7.3.1 Results and Discussion

Picture 3.2 clearly shows that the antibody-linked staining led to whole cell staining. The whole bacterial cell is outlined. Little staining is indicated at the polar flagella. Picture 3.3 shows binding to a mass of displaced exopolysaccharide and to the surface of the cell.



Picture 3.2 Scanning Electron Micrograph of *Ps. putida* antibody stained with Egbert antiserum and a goat anti-rabbit IgG conjugated with gold (which is seen as small black dots)

The electron micrograph shows that the antibody binds onto the surface of the cell. The cell has an outer layer of exopolysaccharide that is known to be highly immunogenic. Little significant binding occurs on the polar flagella.



Picture 3.3 Scanning Electron Micrograph of *Ps. putida* antibody stained with Egbert antisera and a goat anti-rabbit secondary antibody conjugated with gold particles.

The electron micrograph shows antibody binding to a displaced mass of exopolysaccharide and to the surface of the cell.

CHARACTERISATION OF *PSEUDOMONAS*

4.1 AIMS

The aims of this study were to characterise psychrotrophic *Pseudomonas* isolates retrieved from spoiled food and natural environments, such as water and soil. Phenotypic characterisation was initiated with a view to speciating unknown isolates. Once isolate identification was attained the range of cross-reactivities of antibodies produced could be easily assessed (Chapter 3). Molecular techniques were evaluated to ascertain whether the unknown isolates could be speciated. Discrimination, reproducibility, ease of use and rapidity were the main criteria for the molecular techniques investigated.

4.2 INTRODUCTION

The *Pseudomonas* genus has been reclassified in recent years on the basis of rRNA:DNA hybridisation studies that have resulted in a reassessment of the inter-genus relationships (Palleroni *et al.*, 1973). The *Pseudomonas* genus has been divided into five rRNA homology groups, each of which has been assigned to newly formed or pre-existing genera (Tables 1.2 & 1.3). The generic name *Pseudomonas* was reserved for members of rRNA homology group I because the group contained the *Ps. aeruginosa* type strain. The phylogenetic relationship of a number of *Pseudomonas* or *Pseudomonas*-like organisms to those in the established rRNA homology groups is yet unknown (Costas *et al.*, 1992).

Pseudomonads are now known to be distantly related to each other and were grouped together because they shared common characteristics (e.g. Gram negative rods, aerobic, motility by polar flagella and production of catalase). Thus more specialised tests had to be found to differentiate the pseudomonads and a large number of test characters may be necessary for a complete identification (Costas *et al.*, 1992).

In this study *Pseudomonas* spp. were isolated from spoiled food and the environment on selective agar and characterised in the first instance using a short set of morphological and biochemical tests. The short set of tests was performed to confirm that the presumptive *Pseudomonas* isolates were members of the rRNA homology group I. The choice of tests used were applied with reference to the general key for distinguishing between species of rRNA groups I, II and III that was found in Bergey's Manual of Systematic Bacteriology

(Volume 1, 1984)(see also section 4.3.2.3). Groups IV and V *Pseudomonas* species have many properties that set them apart from the members of the other groups. The key two main distinguishing features are: firstly a growth factor requirement, which outside of groups IV and V, is only present in exceptional strains of well characterised species, or in species whose natural relationships are still largely unknown; and secondly, the inability of *Ps. maltophila* and *Ps. vesicularis* to use nitrate as a nitrogen source (Bergey's Manual of Systematic Bacteriology, 1984).

Further biochemical characterisation with a view to speciating or establishing relatedness between the isolates was initiated using commercial kits including Analytical Profile Index 20 NE (API, bioMerieux, France) and Biolog MicroplateT^{M} (Biolog Inc., Hayward, CA, USA). API and Biolog systems have been used to characterise environmental *Pseudomonas* species in many studies with mixed success (Costas *et al.*, 1992; Johnsen *et al.*, 1996; ArnautRolier *et al.*, 1999). Numerical analysis of the nutritional data attained with the Biolog system is discussed in Chapter 5.

Ribotyping and Amplified Ribosomal DNA Restriction Analyis (ARDRA) were the molecular typing methods studied to elucidate relatedness between the isolates.

4.3 PHENOTYPIC CHARACTERISATION OF ENVIRONMENTAL *PSEUDOMONAS*

4.3.1 INTRODUCTION

The isolation of *Pseudomonas* species from psychrotrophically spoiled fish (*Clupea sprattus*) was achieved prior to the commencement of this thesis by Ms Emma Parsons. The isolates found were included in subsequent protocols. *Ps. aeruginosa* NO31 isolated from chicken was obtained from the Food Hygiene Laboratory, Central Public Health Laboratory, Colindale.

4.3.2 METHODS

4.3.2.1 *Pseudomonas* CFC agar

Pseudomonas CFC agar (Oxoid, Basingstoke, UK) comprises an agar base and an antibiotic supplement. The base medium is a modification of King's A medium (King *et al.*, 1954) in which magnesium chloride and potassium sulphate were present to enhance pigment production. The antibiotic supplement (CFC) is added to selectively isolate pseudomonad species generally (rRNA homology groups I-V;(Bridson, 1990). The supplement contains

cetrimide (10μg/ml), fucidin (10μg/ml) and cephaloridine (50μg/ml). The cetrimide facilitates the growth of all pigmented and non-pigmented psychrophilic pseudomonads. The combination of fucidin and cephaloridine aids the selectivity for pseudomonads. The manufacturer's description of *Pseudomonas* CFC agar is that it is a "*specific medium for isolating pseudomonads from chilled foods and processing plants*". Any growth on the agar indicates *Pseudomonas sensu lato* species (rRNA homology groups I-V).

All food and environmental isolates were initially grown on *Pseudomonas* CFC agar and further characterised to establish identity as *Pseudomonas sensu stricto* (rRNA homology group I).

4.3.2.2 Isolation of *Pseudomonas* species

Minced pork (Sutton Bonington Farm, Leicestershire) and pasteurised full fat milk (local shops) were refrigerated (4 °C) for 9 - 15 days. Pork samples (25g) were stomached with MRD (225 ml) and aliquots of the liquid (1 ml) were serially diluted in MRD (9 ml) and spread onto *Pseudomonas* CFC agar.

Aliquots (0.2 ml) of spoiled pasteurised milk were spread onto *Pseudomonas* CFC agar. Soil samples (50g) were mixed with MRD (450 ml) and the mixture was filtered through gauze. The filtrate (0.2 ml) was directly spread onto *Pseudomonas* CFC plates.

Raw milk samples were obtained (Kingston-on-Soar Farm, Leicestershire) from the bulk tank mid flow. Aliquots (1 ml) were serially diluted in MRD and the dilutions (0.1 ml) spread onto *Pseudomonas* CFC agar.

Aliquots of water samples (50-100 ml) collected from the River Soar (Leicestershire), a pond and gutter water were filtered (0.45μm, Gelman Science, USA). The filters were then placed onto the surface of *Pseudomonas* CFC agar and incubated (25°C, 24-48 h).

The agar plates from all other samples were incubated (25°C, 24-48 h) and single colonies were streaked onto a nutrient agar plate, incubated (30°C, 24 h) and stored (4°C) for subsequent testing. All isolates were kept on nutrient agar slopes for long term storage (4°C).

4.3.2.3 Primary physical and biochemical characterisation of *Pseudomonas*

All isolates underwent the following tests: Gram stain, cell morphology, motility (hanging drop and motility agar), oxidase, catalase, oxidation-fermentation of glucose and accumulation of poly-β-hydroxybutyrate (PHB). Methods for each test are described in Chapter 2 or in Cowan and Steel (1974).

All isolates with the profile outlined below were putatively *Pseudomonas* spp. (rRNA homology group I) and retained. Those isolates that did not conform to the profile were rejected. Fluorescence or pigment production was not used as a defining characteristic as the trait, if present, was quickly lost by several isolates on sub-culturing and therefore was not reproducible.

Profile of isolates retained:

- Gram stain -
- Rod shaped +
- Motility +
- Accumulation of PHB -
- Oxidase +
- Catalase +
- Oxidative metabolism of glucose +

4.3.2.4 API 20NE

The API 20 NE system comprises 21 tests. The tests were performed as per manufacturer's instructions and then interpreted using the API 20NE Analytical Profile Index. The API system calculates the Willcox probability, that an unknown strain belongs to a given taxon, expressed as a percentage. The acceptable threshold level for identification can be as low as 80%, but different statements are made as to the level of acceptability of identification. An identification level ≥ 99.9% receives the comment 'excellent identification' and an identification level ≥ 80.0% receives the comment 'acceptable identification'. If the sum of the probabilities for the first three taxa is < 80.0%, then the profile is considered unacceptable and is not listed in the Profile index.

All identification comments given by the Profile index and the presumptive identification were noted for each test isolate. Culture collection strains (Table 4.1) were also screened together with the test isolates.

4.3.3 RESULTS

Approximately 220 environmental and food isolates were picked from the *Pseudomonas* CFC plates. Figure 4.1 shows the growth of *Ps. aeruginosa* (on *Pseudomonas* CFC agar) and *Ps. fluorescens* (on nutrient agar). The production of a green pigment that diffuses into the agar can be seen with the *Ps.aeruginosa* strain. The presumptive *Pseudomonas* spp. underwent primary physical and biochemical characterisation and based on fitting the criteria given in section 4.3.2.3 75 isolates were retained. Figure 4.2 shows typical rod-shaped *Pseudomonas* species as viewed by differential interference microscopy.

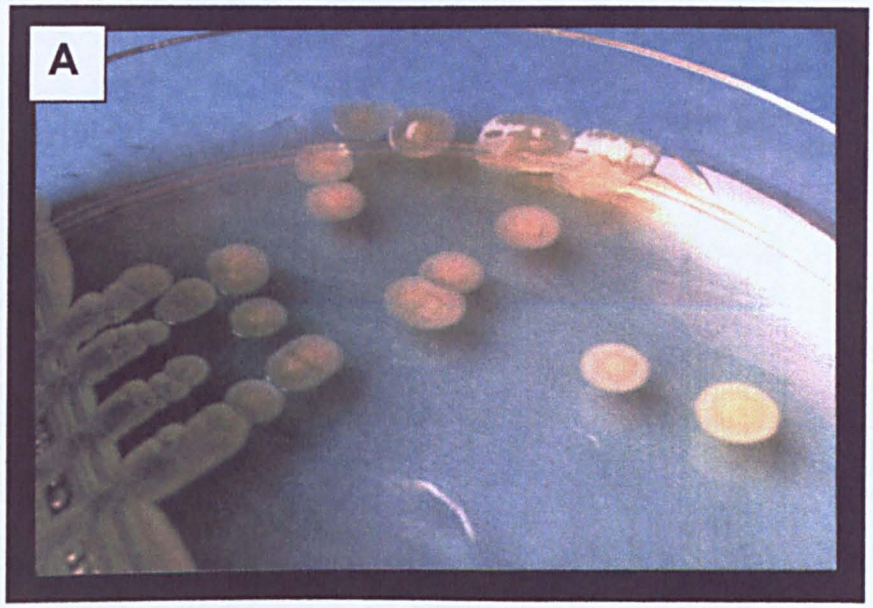
The API 20NE strips identified 69 of the 75 tested environmental isolates as members of the *Pseudomonas* genus (Table 4.1). Three isolates were placed one in each of the genera *Chryseomonas, Xanthomonas* and *Ochrobactrum*. The *Xanthomonas* genus contains pigmented phytopathogens and is currently categorised within the Pseudomonadaceae family. The API 20NE Profile Index gave the comment of 'very good identification' for the *X. maltophila* isolate. Unlike other *Xanthomonas* species, *X.maltophila* (previously *Ps.maltophila*) does not produce xanthomonadin pigments and is oxidase negative (or gives a weak reaction). Based on the oxidase test result produced here, which was clearly positive, the *X.maltophila* isolate may have been misnamed by the API 20 NE system. *Ochrobactrum anthropi* was previously named *Ps. anthropi* and was originally isolated from oil. Due to the Profile index comment of 'doubtful' for *O. anthropi* the identification cannot be considered accurate. *Chryseomonas luteola* (previously named *Ps. luteola*) produces a yellow pigment that was not seen for the isolate tested. The Profile index comment for the isolate was 'unacceptable' and therefore the identification again was not accurate.

Three isolates were identified as *Ps. picketti* (now *Ralstonia picketti*), *Ps. pseudomallei* (now *Burkholderia pseudomallei*) and *Ps. cepacia* (now *Burkholderia cepacia*). All three isolates if correctly identified should accumulate polyhydroxybutyrate as a carbon reserve material. However, all three isolates were negative for PHB, which indicates that they may be misnamed.

Comamonas testosteroni NCTC 10698 (formerly *Ps. testosteroni*; strain 92) was screened with the API 20NE system and the suggested identification for this strain was *Ps. fluorescens*.

However, the Profile Index comment was 'very good ID to genus' and without additional tests, a firm identification could not be made. The culture collection strain was clearly misnamed by the API 20NE system.

Of the 69 isolates that were designated *Pseudomonas* by API 20NE, 14 (20%) were described to the level of genus and 12 (17%) had profiles that were either 'doubtful' or 'unacceptable'. Most of the isolates that were resolved to the level of genus and had doubtful profiles originated from the soil and water environment. Those isolates originating from the food environment were identified with a higher degree of confidence than that seen for the soil and water isolates. The remaining isolates were presumptively identified as *Pseudomonas sensu stricto*. Forty-four percent of the isolates were identified as *Ps. fluorescens*, 13% *Ps. putida* and 8% *Ps. aeruginosa*. *Ps. fluorescens* isolates were ubiquitous and found in all the environments tested. The *Ps. putida* isolates were found in proteinaceous environments such as meat, fish and raw milk. *Ps. aeruginosa* isolates were retrieved from fish, pork and soil in the main.



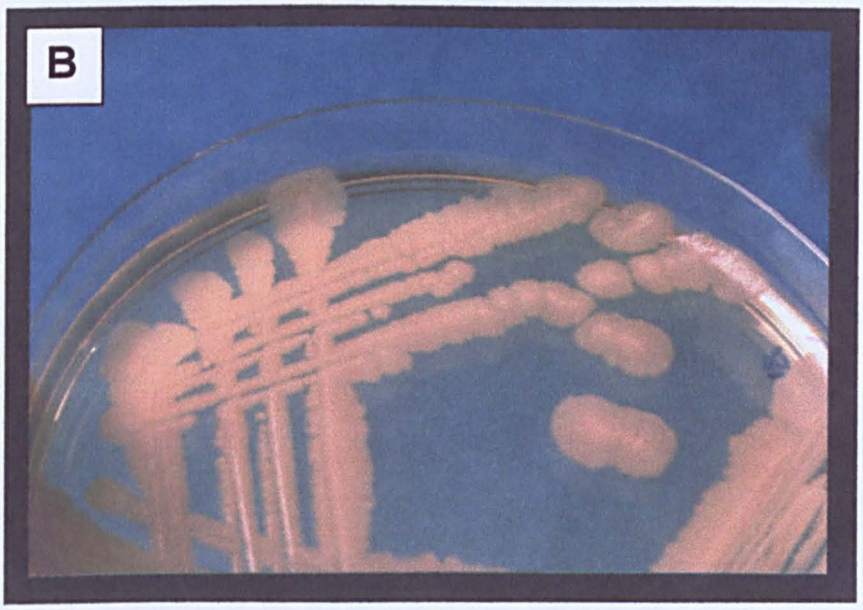
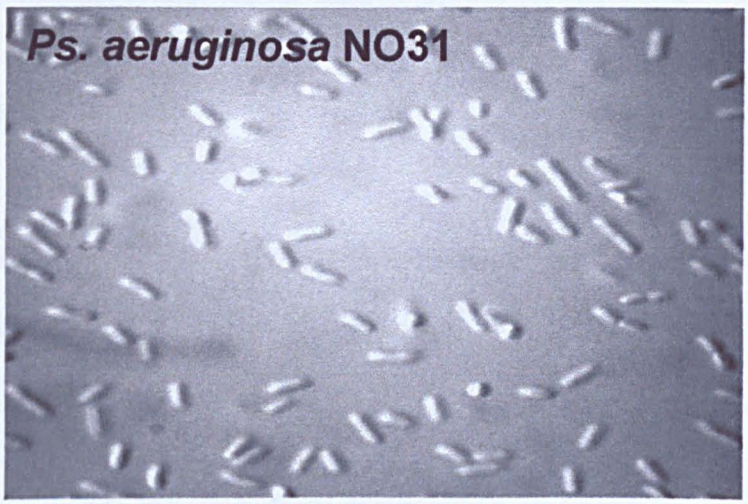


Figure 4.1 Growth of *Pseudomonas* species on solid agar

A: *Pseudomonas aeruginosa* NO31 on *Pseudomonas* CFC agar (30°C, 24h)
B: *Pseudomonas fluorescens* RM1 (raw milk isolate) on nutrient agar (30°C, 24h)



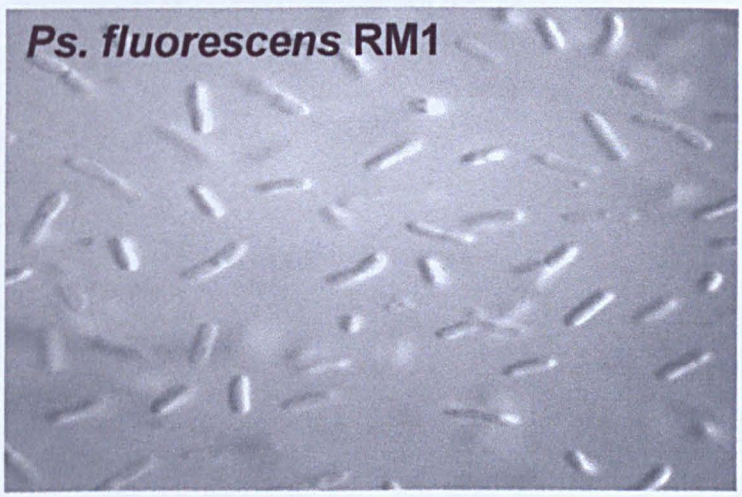


Figure 4.2 Spoilage *Pseudomonas* species viewed using differential interference contrast microscopy

Observed at a magnification x1000

CODE	PRESUMPTIVE ID (API)	API COMMENT	SOURCE
1	*Ps.fluorescens*	VERY GOOD TO GENUS	Fish
2	*Ps.fluorescens*	GOOD ID	Fish
3	*Ps.fluorescens*	ACCEPTABLE	Fish
4	*Ps.fluorescens*	GOOD ID TO GENUS	Fish
5	*Ps.fluorescens*	VERY GOOD TO GENUS	Fish
6	*Ps.fluorescens*	VERY GOOD TO GENUS	Fish
7	*Ps.fluorescens*	GOOD ID	Fish
8	*Ps.fluorescens*	GOOD ID	Fish
9	*Ps.fluorescens*	VERY GOOD TO GENUS	Fish
10	*Ps.putida*	GOOD ID	Fish
11	*Ps.putida*	ACCEPTABLE	Fish
12	*Ps.putida*	VERY GOOD ID	Fish
13	*Ps.putida*	GOOD ID	Fish
14	*Ps.putida*	GOOD ID TO GENUS	Fish
15	*Ps. fluorescens/aureofaciens*	GOOD TO GENUS	Fish
16	*Ps.aeruginosa*	GOOD ID TO GENUS	Fish
17	*Ps.aeruginosa*	GOOD ID	Chicken
19	*Ps.fluorescens*	VERY GOOD ID	Rawmilk
20	*Pseudomonas* spp	VERY GOOD ID TO GENUS	Rawmilk
21	*Ps. picketti*	GOOD ID TO GENUS	Rawmilk
22	*Ps.fluorescens*	GOOD ID	Rawmilk
23	*Ps.putida*	VERY GOOD ID TO GENUS	Rawmilk
24	*Ps.putida*	GOOD ID TO GENUS	Rawmilk
25	*Ps.fluorescens*	GOOD ID TO GENUS	Rawmilk
26	*Pseudomonas* spp	GOOD ID TO GENUS	Rawmilk
27	*Pseudomonas* spp	GOOD ID TO GENUS	Rawmilk
28	*Ps.fluorescens*	GOOD ID	Rawmilk
29	*Ps.fluorescens*	GOOD ID	Rawmilk
30	*Ps.fluorescens*	GOOD ID	Rawmilk
31	*Pseudomonas* spp	DOUBTFUL	Rawmilk
32	*Ps.fluorescens*	VERY GOOD ID	Rawmilk
33	*Ps.fluorescens*	VERY GOOD ID	Past.milk
36	*Ps.fluorescens*	GOOD ID	Past.milk
38	*Pseudomonas* spp	GOOD ID TO GENUS	Past.milk
39	*Ps.fluorescens*	GOOD ID TO GENUS	Past.milk
41	*Ps.aeruginosa*	VERY GOOD TO GENUS	Pork
42	*Ps.aeruginosa*	ACCEPTABLE TO GENUS	Pork
43	*Ps.putida*	UNACEPTABLE	Pork
44	*Ps.fluorescens*	ACCEPTABLE	Pork
45	*Ps.putida*	DOUBTFUL	Pork
46	*Ps.putida*	GOOD ID TO GENUS	Pork

Table 4.1 Screening of environmental isolates with API 20NE

CODE	PRESUMPTIVE ID (API)	API COMMENT	SOURCE
47	*Pseudomonas* spp	DOUBTFUL	Pondwater
48	*Ochrobactrum anthropi*	DOUBTFUL	Pondwater
50	*Ps.fluorescens*	GOOD ID TO GENUS	Pondwater
51	*Ps.fluorescens*	GOOD ID	Pondwater
52	*Pseudomonas* spp	ACCEPTABLE TO GENUS	Pondwater
53	*Ps.fluorescens*	GOOD ID	Pondwater
54	*Ps.fluorescens*	GOOD ID	Gutterwater
55	*Ps.fluorescens*	GOOD ID	Gutterwater
56	*Pseudomonas* spp	DOUBTFUL	Gutterwater
57	*Pseudomonas* spp	DOUBTFUL	Gutterwater
58	*Xanthomonas maltophilia*	VERY GOOD ID	Gutterwater
59	*Ps.fluorescens*	GOOD ID	Gutterwater
61	*Chryseomonas luteola*	UNACCEPTABLE	Riverwater
65	*Pseudomonas* spp	GOOD ID TO GENUS	Riverwater
66	*Pseudomonas* spp	DOUBTFUL	Riverwater
67	*Pseudomonas* spp	EXCELLENT TO GENUS	Riverwater
68	*Pseudomonas* spp	VERY GOOD TO GENUS	Riverwater
69	*Ps.fluorescens*	GOOD ID	Riverwater
70	*Ps.fluorescens*	VERY GOOD ID	Riverwater
71	*Ps.fluorescens*	VERY GOOD TO GENUS	Riverwater
73	*Ps. pseudomallei*	VERY GOOD ID TO GENUS	Riverwater
74	*Ps.fluorescens*	GOOD ID	Riverwater
75	*Ps. mendocina*	EXCELLENT ID	Soil
76	*Pseudomonas* spp	DOUBTFUL	Soil
77	*Ps.aeruginosa*	UNACCEPTABLE	Soil
78	*Ps.fluorescens*	VERY GOOD ID	Soil
79	*Ps.cepacia*	EXCELLENT ID	Soil
80	*Ps.chloroaphis/fluorescens*	EXCELLENT ID TO GENUS	Soil
82	*Ps. chloroaphis*	GOOD ID	Soil
83	*Ps.fluorescens*	UNACCEPTABLE	Soil
84	*Ps. chloroaphis*	GOOD ID	Soil
85	*Ps.fluorescens*	ACCEPTABLE	Soil
86	*Ps.fluorescens*	ACCEPTABLE	Soil
87	*Ps.aeruginosa*	IDENTIFICATION NOT VALID	Soil
Culture collection strains			
88	*Ps.aeruginosa*	VERY GOOD ID	NCTC 10332
91	*Ps.fluorescens (Ps.fluorescens)*	EXCELLENT ID	NCTC 10038
92	*Ps. fluorescens (Ps.testosteroni)*	VERY GOOD ID TO GENUS	NCTC 10698

Table 4.1 Continued Screening of environmental isolates with API 20NE

4.3.4 Discussion

The use of the API 20 NE system enabled the majority (~ 80%) of the food and environmental isolates to be identified. The API test strips were standardised, easy to use and more rapid than classical tests. However, there are some concerns about the API identifications. One of the culture collection strains and presumptively three isolates were misnamed (total 5%) and approximately 19% were not speciated. The misnaming of the isolates casts a doubt about the reliability of the identifications for all the other test organisms. Difficulties with identifying environmental *Pseudomonas* isolates using API 20NE have been reported by other workers (Morais *et al.*, 1997). Since the initiation of these investigations, the manufacturer's of the API 20NE have produced a Profile Index database that incorporates the recent name changes of *Pseudomonas sensu lato* species. The use of the new Profile Index database may have influenced the outcome of some of the identifications given in this study.

4.4 Cross-reactions of antisera with environmental *Pseudomonas*

4.4.1 Introduction

The *Pseudomonas* isolates used to raise antisera came from specific environmental sources (fish, chicken, milk and raw milk). However, *Pseudomonas* spp. are ubiquitous in the environment and consequently a vast reservoir of the organisms exist that are capable of contaminating food. To be useful, an antiserum needs to cross-react with its target organism regardless of source. Thus, the aims of this study were to investigate how widely the epitopes recognised by the characterised *Pseudomonas* antisera (Chapter 3) were distributed in the environment. The environmental isolates examined were those retrieved from food spoiled at refrigeration temperature (milk & pork), raw milk, a natural watercourse (River Soar, Leicestershire) and the soil and characterised in the previous section. Additionally, the antibodies raised against *Pseudomonas* species should confirm the identification for those isolates speciated by API 20NE and may aid the identification of those isolates not speciated.

4.4.2 METHODS

Environmental isolates were grown overnight in BHI broth (18h, 30°C) and dot blots were performed on unwashed cells as given in Section 2.11. All 75 environmental isolates shown in Table 4.1 were assayed. The culture density for each test sample was standardised by eye to that of the test cell suspension with the lowest turbidity. Seven antisera were assayed at the optimum working dilution for each (Chapter 3) and non-immune serum was used as a control. A positive control (consisting of a strain against which the antiserum was raised) was included on each filter. All antibody cross-reactions were recorded as positive if the reaction was considered stronger than the non-immune serum. Where reaction differences were borderline these were still scored as positive.

4.4.3 RESULTS

Data from selected isolates (56/75) that represented each environment are presented. The results obtained from the selected isolates applied across all strains tested. The cross-reactions of environmental *Pseudomonas* with each antiserum are shown in Figure 4.3. The data from the dot blots are tabulated in Table 4.2. The percentage cross-reactivity of each antiserum with isolates from a specific source is shown in Table 4.3.

4.4.4 DISCUSSION

The cross-reactions of *Pseudomonas* antisera were influenced by the origin of the organism as well as the species of the organism. For example, Egbert antiserum (raised against *Ps. putida* from fish) cross-reacted with 12/26 presumptive *Ps. fluorescens*, 3/5 *Ps. putida* and 4/4 *Ps. aeruginosa*. Relating Egbert antiserum's cross-reactivity to source, cross-reactions were seen with 11/15 water, 6/6 pork, 4/17 raw milk, 0/5 milk and 4/13 soil isolates. In general few cross-reactions were seen with milk and soil isolates whilst Egbert antiserum cross-reacted well with isolates from water and pork.

Xerxes was raised against *Pseudomonas* spp. from raw milk and cross-reacted with 100% raw milk and pork isolates irrespective of the identity of the isolates (Table 4.3). Xerxes and Xavier cross-reacted widely with isolates from all habitats indicating broad-spectrum specificity. The antisera raised to isolates from fish or chicken cross-reacted well with the pork isolates but did not cross-react with milk isolates with the exception of Edward antiserum. The milk isolates were mainly *Ps.fluorescens* and Edward was raised against

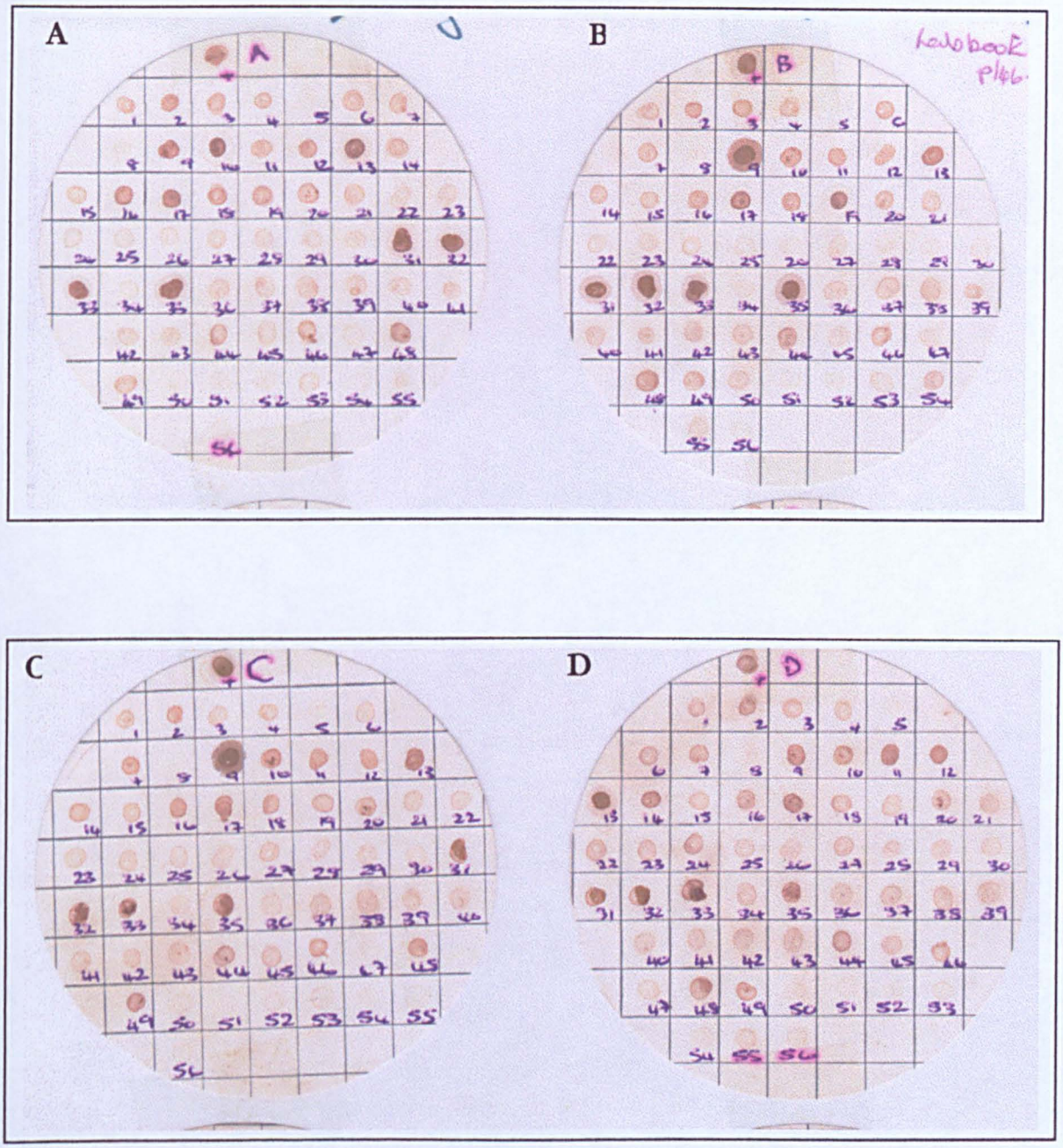
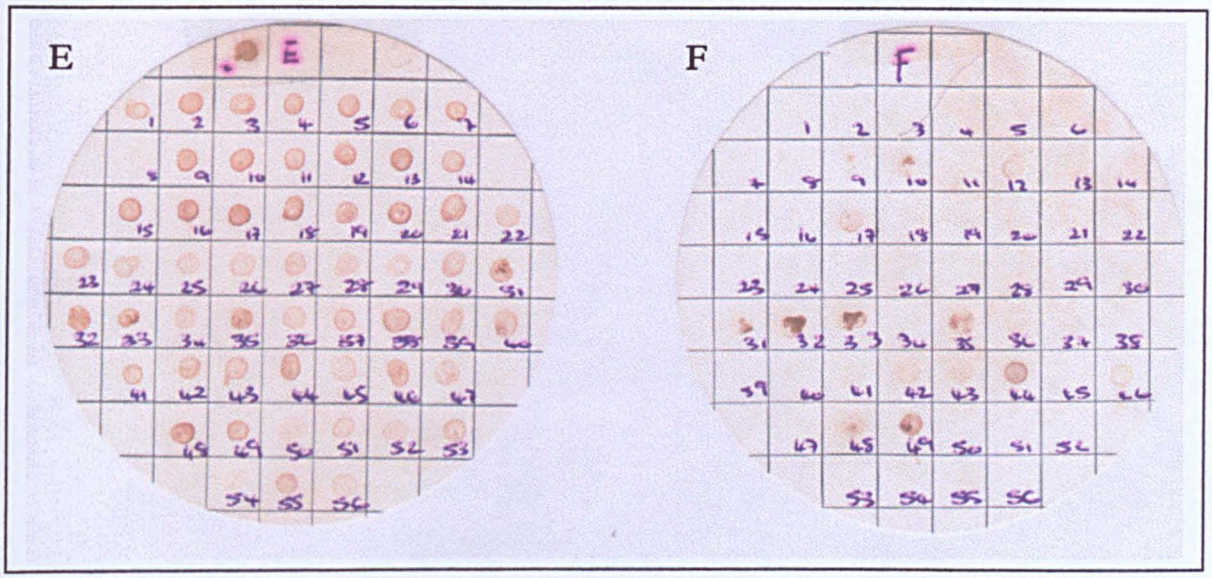


Figure 4.3 Cross-reactions of environmental *Pseudomonas* with each antiserum

Antiserum designation: A = Egbert (*Ps. putida*) B = Edward (*Ps. fluorescens*)
C = Entwistle (*Ps. aeruginosa*) D = Eamon (*Ps. alcaligenes*)

Identification for each isolate (1-56) is given in Table 4.2. At the top of each filter a positive control for each antiserum was added.



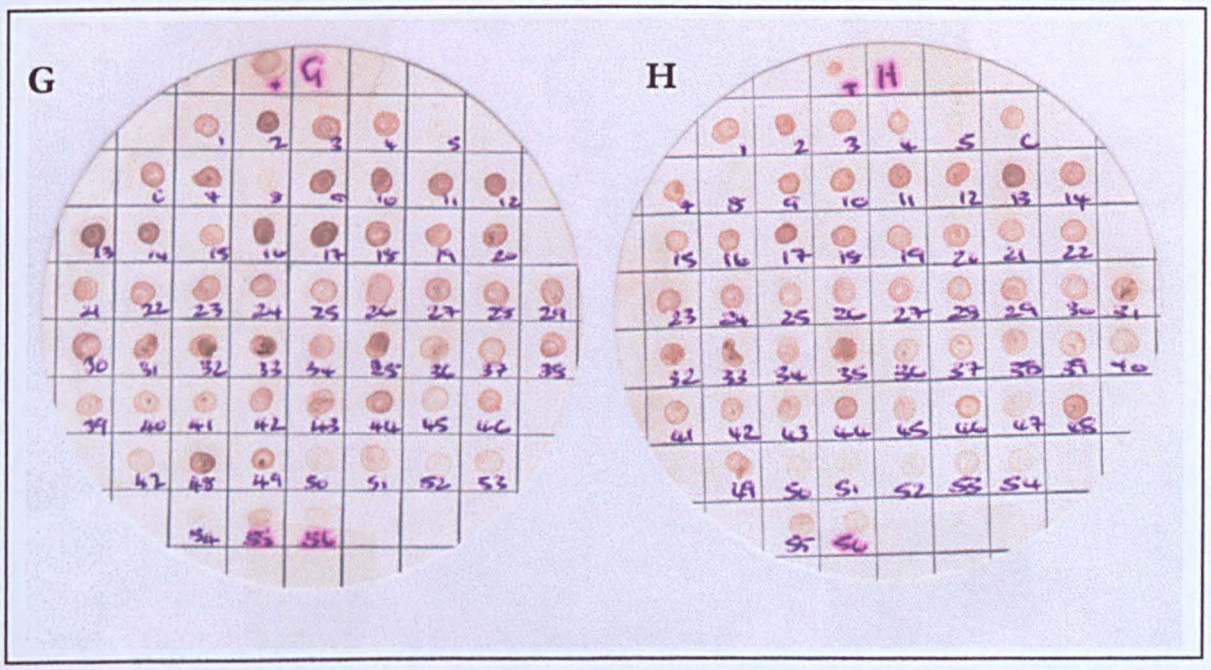


Figure 4.3 (continued) Cross-reactions of environmental *Pseudomonas* with each antiserum

Antiserum designation: E = Xtra (*Ps. aeruginosa*) F = Non Immune Serum

G = Xerxes (*Ps. putida*) H = Xavier (*Ps. fluorescens*)

Identification of each isolate (1-56) is given in Table 4.2. At the top of each filter a positive control for each antiserum was added.

Table 4.2 Cross-reactions of environmental *Pseudomonas* with each antiserum

Organisms (API identification)	Source	Cross-reactions of each antiserum							
		Egbert *Ps.putida*	Edward *Ps.fluorescens*	Entwistle *Ps.aeruginosa*	Eamon *Ps. alcaligenes*	Xtra *Ps.aeruginosa*	Non-immune serum (NIS)	Xerxes *Ps.putida*	Xavier *Ps. fluorescens*
1 Ps. fluorescens	WATER	+						+	+
2 Ps. fluorescens	WATER	+	+	+	+	+		++	+
3 Pseudomonas spp.	WATER	+	+		+			+	+
4 Pseudomonas spp.	WATER	+						+	+
5 Xanthomonas maltophila	WATER					+			
6 Ps. fluorescens	WATER	+	+					+	+
7 Pseudomonas spp.	WATER	+		+		+		+	+
8 Chryseomonas luteus	WATER								
9 Ps. aeruginosa	PORK	++	++	++	++	+		++	+
10 Ps. aeruginosa	PORK	++	+	+	+	+		++	+
11 Ps. putida	PORK	+	+	+	+	+		++	+
12 Ps.fluorescens	PORK	+		+	+	+		++	+
13 Ps. putida	PORK	++	++	++	++	+		++	++
14 Ps. putida	PORK	+		+				++	+
15 Ochrobacter anthropi	WATER			+		+			+
16 Pseudomonas spp.	WATER	++	+	+	+	+		++	+
17 Ps. fluorescens	WATER	++	++	+	++	+		++	++
18 Ps. fluorescens	WATER	+	+	+	+	+		+	+
19 Pseudomonas spp.	WATER	+	++		++	+		+	+
20 Ps. fluorescens	WATER	+		+		+		+	+
21 Pseudomonas spp.	WATER					+		+	+
22 Ps. fluorescens	R.MILK							+	+
23 Pseudomonas spp.	R.MILK							+	+
24 Ralstonia picketti	R.MILK							+	+
25 Ps. fluorescens	R.MILK							+	
26 Ps.putida	R.MILK							+	+
27 Ps. putida	R.MILK							+	+
28 Ps. fluorescens	R.MILK							+	+

blank = negative + = positive ++ = strong positive

Table 4.2 (continued) Cross-reactions of environmental *Pseudomonas* with each antiserum

Organisms (API identification)	Source	Cross-reactions of each antiserum							
		Egbert *Ps.putida*	Edward *Ps.fluorescens*	Entwistle *Ps.aeruginosa*	Eamon *Ps.alcaligenes*	Xtra *Ps.aeruginosa*	Non-immune serum (NIS)	Xerxes *Ps.putida*	Xavier *Ps.fluorescens*
29 Pseudomonas spp.	R.MILK							+	+
30 Pseudomonas spp.	R.MILK							+	++
31Ps. fluorescens	R.MILK	++	++	++	++	+		+	++
32 Ps. fluorescens	R.MILK	++	++	++	++	+	+	++	++
33 Ps. fluorescens	R.MILK	++	++	++	++	+	+	++	
34 Pseudomonas spp.	R.MILK							+	++
35 Ps. fluorescens	R.MILK	++	++	++	++	+	+	++	
36 Ps. fluorescens	R.MILK							+	
37 Ps. fluorescens	R.MILK		+					+	
38 Ps. fluorescens	R.MILK		+			+		++	+
39 Ps. fluorescens	MILK		+						
40 Ps. fluorescens	MILK								
41 Pseudomonas spp.	MILK								+
42 Ps. fluorescens	MILK		+					+	+
43 Ps. fluorescens	MILK							+	+
44 Ps. mendocina	SOIL	+	+	+	+	+	+	+	+
45 Pseudomonas spp.	SOIL								
46 Ps. aeruginosa	SOIL	+	+	+	+	+		+	+
47 Ps. fluorescens	SOIL								
48 Ps. fluorescens	SOIL	++	+	+	+	+		++	++
49 Ps. aeruginosa	SOIL	+	+	+	+	+	+	+	+
50 Ps. fluorescens	SOIL		+						
51 Burkholderia cepacia	SOIL								
52 Ps. chloraphis	SOIL								
53 Ps.chloroaphis/fluorescens	SOIL								
54 Ps. fluorescens	SOIL		+			+			
55 Ps. chloroaphis	SOIL								
56 Ps.fluorescens	SOIL								

SOURCE	SAMPLE NUMBER	% CROSS-REACTIONS OF EACH ANTISERUM							
		F	F	F	F	C		RM	RM
		EGBERT *Ps.putida*	EDWARD *Ps.fluorescens*	ENTWISTLE *Ps.aeruginosa*	EAMON *Ps.alcaligenes*	XTRA *Ps.aeruginosa*	NIS	XERXES *Ps.putida*	*XAVIER Ps.fluorescens*
WATER	15	73	47	47	40	67	0	80	87
PORK	6	100	67	100	83	83	0	100	100
RAW MILK	17	24	35	24	24	29	18	100	70
MILK	5	0	60	0	0	0	0	40	80
SOIL	13	31	46	31	31	38	15	31	31

Table 4.3 Percentage cross-reactions of *Pseudomonas* antisera with environmental *Pseudomonas* spp.

Origin of isolate each antiserum was raised against F = fish C= chicken RM= raw milk

Ps.fluorescens. The cross-reactivities of Edward antiserum in this case were highly related to the species of the organism and not the environmental source they came from.

The association of antiserum cross-reactivity with isolate environment rather than species against which it was raised may reflect underlying fundamental differences in the phenotypic component being characterised. Antibodies may detect the phenotypic expression of an organism associated with living in a given environment and this may be distinct from that part of the phenotype characterised for taxonomic designations. *Pseudomonas* species are classified according to their genotype (taxonomically relevant phenotypic characteristics are used to aid identification) whereas antibodies recognise the phenotype expressed in a given environment. Different species or genera of organisms may adapt to living in the same environment or niche by displaying similar necessary phenotypic characteristics hence antibodies appear to pick up environmentally related populations not solely the species against which it was raised. In halotolerant species, widely diverse taxonomic groupings have been shown to express similar phenotypic properties when isolated from the same environment (Zeynep Sonal personal communication). In general, antibodies used to characterise natural populations of a given species should therefore be raised against organisms taken from the environment to be assessed. Because there was no clear association of cross-reactivity with species the antibodies were unable to substantiate the presumptive identifications given by the API 20NE system. Molecular typing methods based on 16S rRNA were therefore evaluated to see if they could aid speciation of the environmental isolates.

4.5 MOLECULAR CHARACTERISATION OF *PSEUDOMONAS*

4.5.1 INTRODUCTION

Genomic fingerprinting techniques such as ribotyping, and Amplified Ribosomal DNA Restriction Analysis (ARDRA, Figure 4.4) have been used successfully for taxonomic classification, including identification, of many bacterial species (Bingen *et al.*, 1994; Heyndrickx *et al.*, 1996). Ribosomal DNA genes are ubiquitous and highly conserved in Eubacteria (Bingen *et al.*, 1994).

Ribotyping examines the polymorphisms within and surrounding ribosomal DNA genes (Grimont & Grimont, 1986; Stull *et al.*, 1988). The term polymorphism relates to the differences that occur in the nucleotide sequences of isolates of the same species.

ORIGIN 5' end of mature rRNA. **A**

1 aaattgaaga gtttgatcat ggctcagatt gaacgctggc ggcaggccta acacatgcaa
61 gtcgaacggt aacaggaaga agcttgctct ttgctgacga gtggcggacg ggtgagtaat
121 gtctgggaaa ctgcctgatg gaggggata actactggaa acggtagcta ataccgcata
181 acgtcgcaag accaaagagg gggaccttcg ggcctcttgc catcggatgt gcccagatgg
241 gattagctag taggtggggt aacggctcac ctaggcgacg atccctagct ggtctgagag
301 gatgaccagc cacactggaa ctgagacacg gtccagactc ctacgggagg cagcagtggg
361 gaatattgca caatgggcgc aagcctgatg cagccatgcc gcgtgtatga agaaggcctt
421 cgggttgtaa agtactttca gcggggagga agggagtaaa gttaatacct ttgctcattg
481 acgttacccg cagaagaagc accggctaac tccgtgccag cagccgcggt aatacggagg
541 gtgcaagcgt taatcggaat tactgggcgt aaagcgcacg caggcggttt gttaagtcag
601 atgtgaaatc cccgggctca acctgggaac tgcatctgat actggcaagc ttgagtctcg
661 tagaggggg tagaattcca ggtgtagcgg tgaaatgcgt agagatctgg aggaataccg
721 gtggcgaagg cggccccctg gacgaagact gacgctcagg tgcgaaagcg tggggagcaa
781 acaggattag ataccctggt agtccacgcc gtaaacgatg tcgacttgga ggttgtgccc
841 ttgaggcgtg gcttccggag ctaacgcgtt aagtcgaccg cctggggagt acggccgcaa
901 ggttaaaact caaatgaatt gacgggggcc cgcacaagcg gtggagcatg tggtttaatt
961 cgatgcaacg cgaagaacct tacctggtct tgacatccac ggaagttttc agagatgaga
1021 atgtgccttc gggaaccgtg agacaggtgc tgcatggctg tcgtcagctc gtgttgtgaa
1081 atgttgggtt aagtcccgca acgagcgcaa cccttatcct ttgttgccag cggtccggcc **B**
1141 gggaactcaa aggagactgc cagtgataaa ctggaggaag gtggggatga cgtcaagtca
1201 tcatggccct tacgaccagg gctacacacg tgctacaatg gcgcatacaa agagaagcga
1261 cctcgcgaga gcaagcggac ctcataaagt gcgtcgtagt ccggattgga gtctgcaact
1321 cgactccatg aagtcggaat cgctagtaat cgtggatcag aatgccacgg tgaatacgtt
1381 cccgggcctt gtacacaccg cccgtcacac catgggagtg ggttgcaaaa gaagtaggta
1441 gcttaacctt cgggagggcg cttaccactt tgtgattcat gactggggtg aagtcgtaac
1501 aaggtaaccg taggggaacc tgcggttgga tcacctcctt a 3' end

Figure 4.4 The sequence of *E.coli* 16S rDNA with the ARDRA primers binding sites highlighted

The highlighted areas show the location of the forward (A) and reverse (B) ARDRA primers binding sites within the nucleotide sequence of *E.coli* 16S rDNA. The primers bind to the conserved regions of the 16S rDNA and act as universal primers for other Eubacterial rDNA genes. The E.coli 16S rDNA sequence (accession number J01859) was obtained from the National Center for Biotechnology Information, National Library of Medicine, Bethesda, USA.

Size of PCR product = Reverse Primer 1065R (20 bp) - Forward primer 41F (20 bp)

= 1024 base pairs (bp)

The polymorphisms within the rDNA are species specific and hence can be used as a classification tool.

Ribotyping involves cleaving genomic DNA with endonucleases and separating the genomic digest by electrophoresis on an agarose gel. The resulting fragment pattern appears as a smear due to the large number of bands generated. The genomic digest is then transferred onto a membrane for easy handling. Particular bands are highlighted using a labelled probe, such as rDNA or copy DNA (DNA synthesised directly from rRNA using the enzyme reverse transcriptase). The visualisation of the probed fragments leads to a reduced number of bands and can yield a species-specific "fingerprint".

ARDRA is a PCR technique that amplifies regions of DNA via 16S rDNA primers (PCR ribotyping). The amplified products are digested with restriction enzymes and separated by electrophoresis on an agarose gel. The technique relies on there being key differences within the rDNA which are species specific, hence the digested ARDRA products form a "fingerprint" that can be used to differentiate between bacterial species (Smit *et al.*, 1997; Vandamme *et al.*, 1996). The ARDRA technique is a rapid method especially when compared to ribotyping as the PCR amplification, restriction digest and gel electrophoresis take only 7 hours whilst ribotyping can take up to 3 days.

4.5.2 RIBOTYPING

The preparation of genomic DNA was achieved using the cetyltrimethylammonium-bromide (CTAB) method as described by Ausubel *et al.* (1989) with the omission of the cesium chloride steps (Section 2.18). The CTAB method can be of value with strains that produce DNAase (Owen & Hernandez, 1993). Restriction digest of the genomic DNA was attained with the endonuclease *Eco*RI which is a six base cutter recognising the sequence 5'GAATTC3' (Section 2.21). The genomic digest was separated by electrophoresis on a 0.8% agarose gel and run at 30V overnight (Section 2.19). The DNA fragments (smear) were transferred onto a nylon membrane (Southern Blotting; Section 2.21).

Copy DNA (cDNA) riboprobe was synthesised from 16S and 23S rRNA of *E.coli* using reverse transcriptase (Section 2.21). A digoxigenin (DIG) label was incorporated into the probe during production. Digoxigenin bound to deoxyuridine triphosphate (dUTP) is incorporated into the DNA (Pollard - Knight, 1990). The digoxigenin labelled probes are

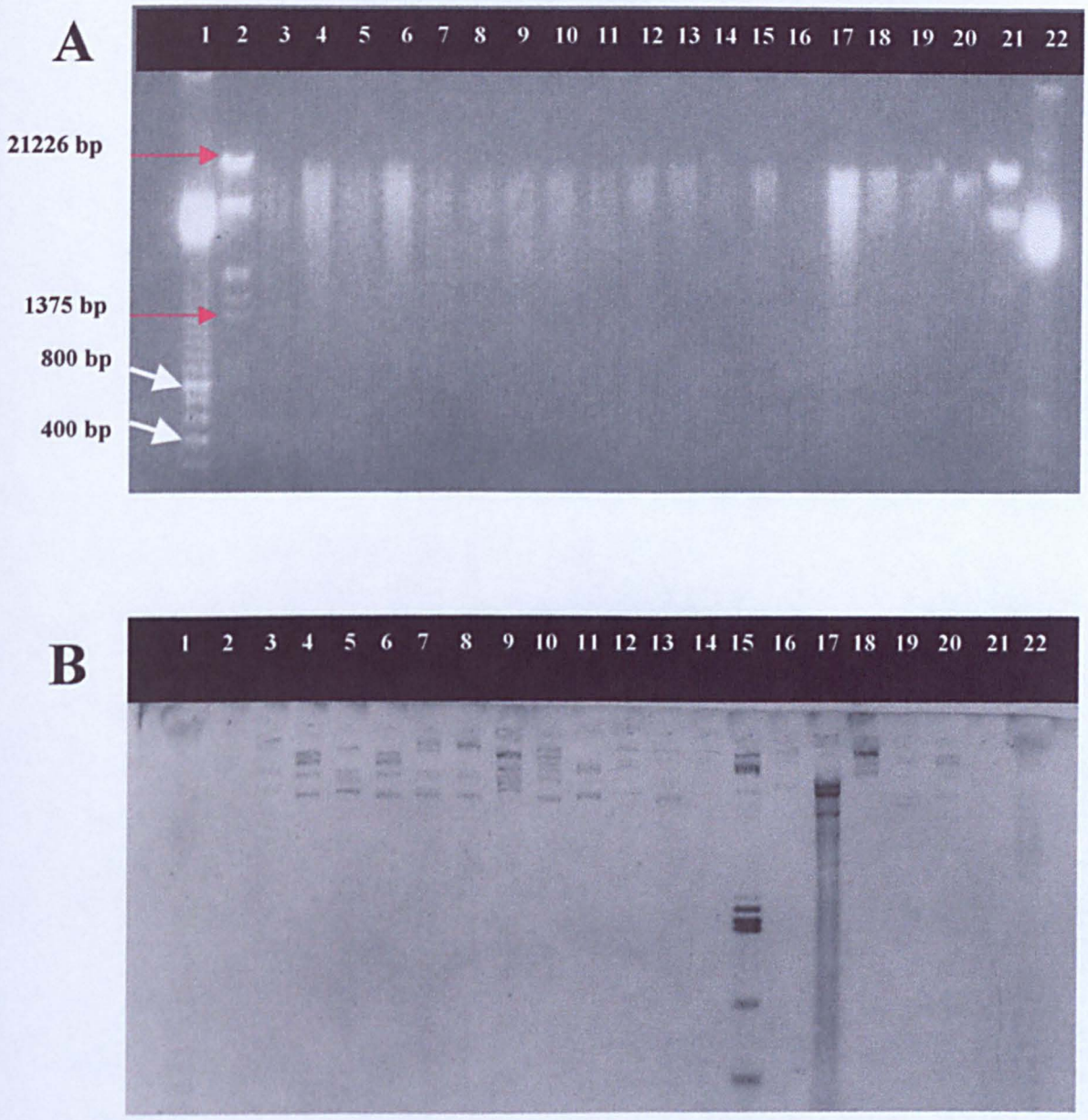
detected by sheep anti-digoxigenin polyclonal antibody conjugated to alkaline phosphatase. The steroid hapten digoxigenin occurs naturally in digitalis plants but is not present in most tissues thus few non-specific binding events should occur (Pollard & Knight, 1990). The probed nylon membranes were developed and the patterns from each bacterial isolate were compared.

Selected food and environmental isolates previously examined by API 20NE and culture collection strains were used to evaluate the ribotyping procedure as a means of confirming or suggesting the identification of strains. The organisms labelled Environmental 5, 6 & 8 were laboratory isolates that originated from water and were confirmed as *Ps. putida* by API 20 NE.

4.5.3 Results and Discussion

Figure 4.5A shows the digested chromosomal DNA separated by electrophoresis on an agarose gel. The digests appear as a long smear running down the gel. Figure 4.5B shows the ribotyping patterns attained for each bacterial DNA sample hybridised with the dig-labelled riboprobe. The distance travelled by each fragment was noted and can be seen in Table 4.4.

The *Ps. putida* samples tested (lanes 3-8) fell into three pattern groups. The lab strain and strains EM17 and EM20 (fish isolates) were all identical to each other as were EM11 and EM14 (fish isolates). The riboprint for EM12 (fish isolate) was distinct from the other *Ps. putida* patterns but had three of its five bands in common with EM11 and EM14. *Ps. putida* is known to be a heterogeneous species that can be sub-divided into two biovars (Stanier *et al.*, 1966). Hence the two main groups here may represent these biovars with EM12 being a variant of the group containing EM11 and EM14. The three environmental isolates (lanes 17-19) were presumptively identified as *Ps. putida* by API 20NE however, with the



Lane		Lane	
1	100bp ladder	12	*Ps. aeruginosa* - NO31
2	λ *Eco*RI / *Hind*III marker	13	*Ps. alcaligenes* - NCTC 10367
3	*Ps. putida* - lab strain	14	*Ps. alcaligenes* - PS 537
4	*Ps. putida* - EM11	15	*E. coli* - FSAC EJ1a
5	*Ps. putida* - EM12	16	*A. junii* - NCTC 12153
6	*Ps. putida* - EM14	17	*Ps. putida* Environmental-5
7	*Ps. putida* - EM17	18	*Ps. putida* Environmental-6
8	*Ps. putida* - EM20	19	*Ps. putida* Environmental-8
9	*Ps. fluorescens* - NCTC 10038	20	*Ps. fluorescens* Milk Isolate-36
10	*Ps. fluorescens* - EM36	21	λ *Eco*RI / *Hind*III marker
11	*Ps. aeruginosa* - NCTC 10332	22	100bp ladder

Figure 4.5 Ribotyping of *Pseudomonas* isolates

A: Genomic DNA digest with *Eco*R1 (37°C for 2 h). The fragments were run overnight (30V) on an agarose gel (0.8%) containing ethidium bromide (0.5mg/ml). The DNA fragments appear as a smear on the gel

B: Riboprint produced after digested genomic DNA transferred onto a nylon membrane and probed with a DIG labelled cDNA probe from *E.coli* 16S and 23S rRNA.

Organisms	Distance travelled by each band (mm)									
	1	2	3	4	5	6	7	8	9	10
Ps. putida Lab strain	2	3	6	7	9					
Ps. putida EM11	3	5	6	8						
Ps. putida EM12	3	5.5	6	7	8					
Ps. putida EM14	3	5	6	8						
Ps. putida EM17	2	3	6	7	9					
Ps. putida EM20	2	3	6	7	9					
Ps. fluorescens NCTC 10038	3.5	5	6	6.5	7.5					
Ps. fluorescens EM36	2	3	4	5	5.5	8				
Ps. aeruginosa NCTC 10332	4	5	8							
Ps.aeruginosa NO31	2	3.5	4	7.5						
Ps. alcaligenes NCTC 10367	2	3.5	8							
Ps. alcaligenes PS537	2	3.5	8							
E.coli EJ1a	2	3.5	4.5	7	20	22	24	25	34	44
A. junii NCTC 12153	2	2.5	3.5	7	7.5					
Environmental 5	6.5	7.5	10							
Environmental 6	2	3.5	4							
Environmental 8	2.5	3	5	8						
Milk isolate- 36	2	3	5	6.5						

Table 4.4 Distance travelled by each fragment within the riboprint (mm)

exception of Environmental- 8 which had bands in common with both *Ps. putida* groups, little resemblance to the other *Ps. putida* isolates was seen.

The two *Ps. alcaligenes* tested (one a culture collection strain, lanes 13 & 14) produced identical riboprints. The riboprints from the two *Ps. fluorescens* (lanes 9 & 10) were very different from each other sharing one common band. Likewise, the two *Ps.aeruginosa* (lanes 11 & 12) produced patterns that were dissimilar, with one common band. *Ps. aeruginosa* produced three or four bands when cut with *Eco*RI. Grattard *et al.* (1994) also found that 40 strains of *Ps. aeruginosa* isolated from a hospital environment produced three or four bands on the riboprint using *Eco*RI with eight different banding patterns attained. Grattard and co-workers used a more discriminatory restriction endonuclease, *Pvu*I (restriction site CGATCG), with the same 40 strains and produced 29 banding patterns (from seven to twelve bands).

Ps. aeruginosa is known to be a homogeneous group with little variation within the species. However, the two strains produced different patterns and, as indicated above, larger groups of strains can produce diverse patterns. The variation in the riboprints between the type strain of *Ps.fluorescens* and the EM36 isolate might suggest the latter isolate was misnamed. However, *Ps. fluorescens* is a heterogeneous group comprising five biovars (Stanier *et al.*, 1966) and therefore variation in ribotype patterns within the species may be expected. Thus the production of differing patterns here may not preclude a species relationship. A larger number of strains needs to be compared. Alternatively, it could be argued that the pattern of bands produced by the *Eco*RI endonuclease was not appropriate to yield species discerning riboprints due to the small number of bands produced. Milk isolate-36 (*Ps. fluorescen*, Table 4.1) had three (out of four) bands in common with the *Ps. fluorescens* EM36 and so could be the same species. However the uncertainty of how many bands need to be identical to constitute being in a species makes such an association dubious. Indeed isolate Environmental 6 (*Ps. putida*) showed as many bands in common with *A.junii* as it did with any of the *Pseudomonas* species.

In previous studies ribotyping has been shown to be a powerful tool that can resolve bacterial isolates into sub-species giving each specimen a unique pattern. The number of samples tested in this study was not sufficient to determine absolute relatedness of the samples tested. The sensitivity of ribotyping depends greatly on the endonucleases used. Altwegg & Mayer (1989) demonstrated that strains of *Salmonella* could be differentiated with endonucleases *Pst*I or *Sma*I but not with *Eco*RI. The non-*Pseudomonas* strains produced

patterns that were distinctly different to those produced by the *Pseudomonas* isolates and known strains. As mentioned previously the riboprints from the *Pseudomonas* isolates using *Eco*RI produced between three and six bands. The bands produced from the *Pseudomonas* isolates represent large pieces of DNA indicating that few *Eco*RI restriction sites were distributed throughout the *Pseudomonas* genome. The molar percentage ratio of guanidine:cytosine (G:C) in the *Pseudomonas* genome is between 57-70 (Palleroni, 1984). A restriction endonuclease that cuts at sites on the genome containing a GC nucleotide pairing or applying multiple cutting enzymes may yield more fragments that hybridise with the riboprobe after digestion. The broader pattern obtained would probably be more discerning such that sub-species variation may be more clearly elucidated.

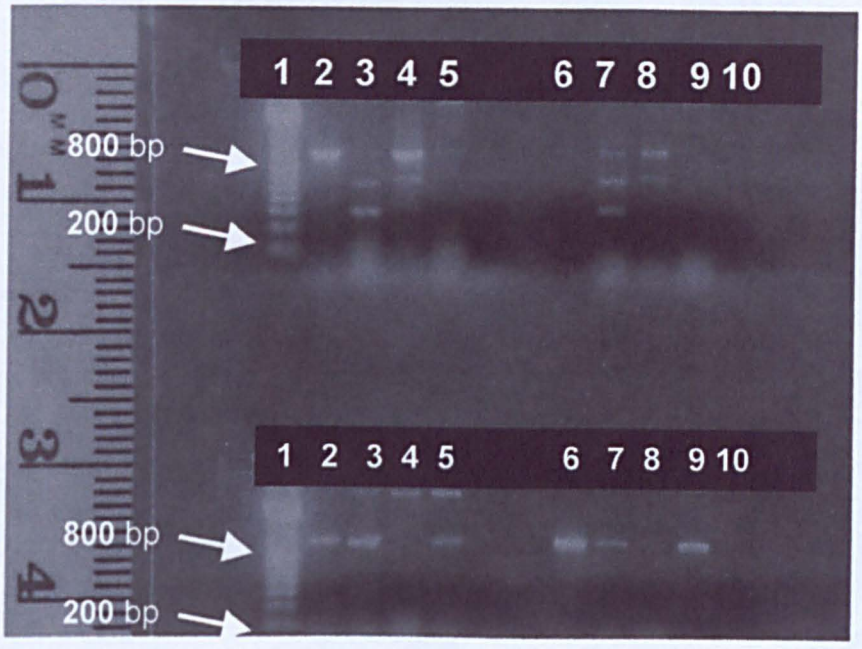
The ribotyping procedure is time-consuming and labour intensive to perform (due to the requirement for Southern blotting) and without the aid of appropriate computer-software may be time-consuming to analyse. However, the banding patterns produced by ribotyping can be definitive i.e. high typeability and reproducibility (Blanc *et al.*, 1993).

4.5.4 ARDRA PCR

Freshly grown (18h, 30°C) single bacterial colonies on solid agar (BHI) were aseptically removed with a toothpick and resuspended in sterile reverse osmosis water (100 µl). The cell suspensions were boiled for 10 min and added (2 µl) to the PCR reaction mix as described in Section 2.22. The PCR conditions used are outlined in Sections 2.22.1 & 2.22.2. A negative control contained all reagents except the template DNA. Environmental and food isolates and standard culture strains (35) were used to evaluate ARDRA PCR.

4.5.5 Results and Discussion

After amplification of the 16S rDNA the expected gene product was a single 1024 base pair band (Figure 4.4). Approximately a third of the isolates (10/35) produced multiple small bands, a third of the samples (13/35) produced no bands at all and the expected band (~1kbp) was produced by the remaining isolates (12/35).



Lane

1. 100 bp ladder
2. *Ps. alcaligenes* EM9
3. *Ps.putida* EM11
4. *Ps.fluorescens* EM31
5. *Ps. aeruginosa* NO31
6. *Ps. alcaligenes* EM9
7. *Ps.putida* EM11
8. *Ps.fluorescens* EM31
9. *Ps. aeruginosa* NO31
10. Control - no cells

TOP GEL - STANDARD ARDRA PCR

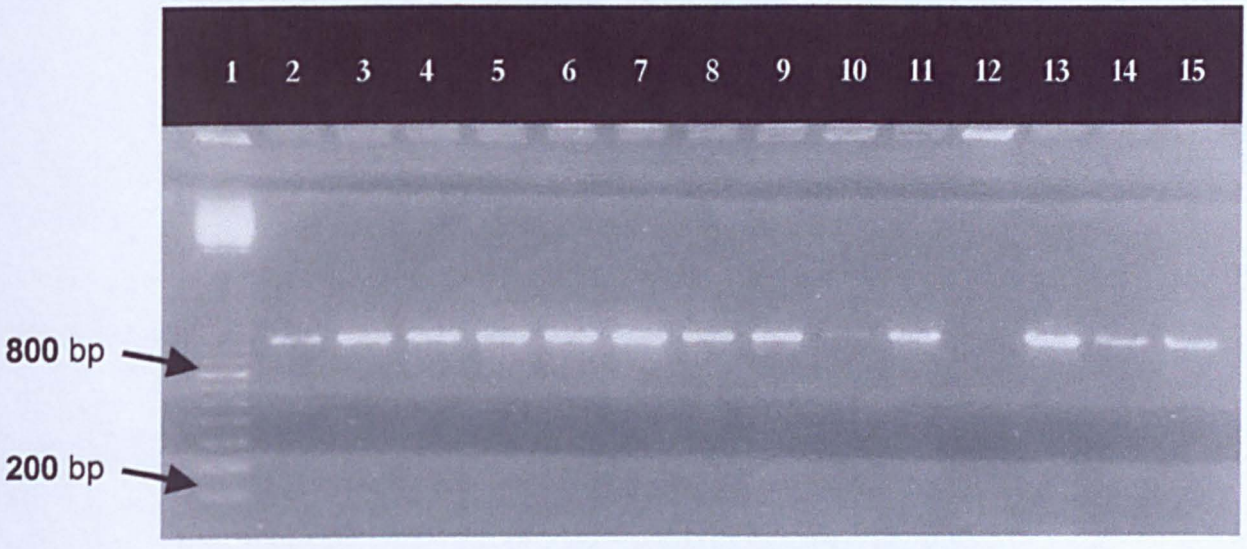
Lane	Addition of 5% glycerol
2-5	-
6-10	+

BOTTOM GEL - TOUCHDOWN PCR

Lane	Addition of 5% glycerol
2-5	-
6-10	+

Figure 4.6 Optimisation of ARDRA PCR conditions

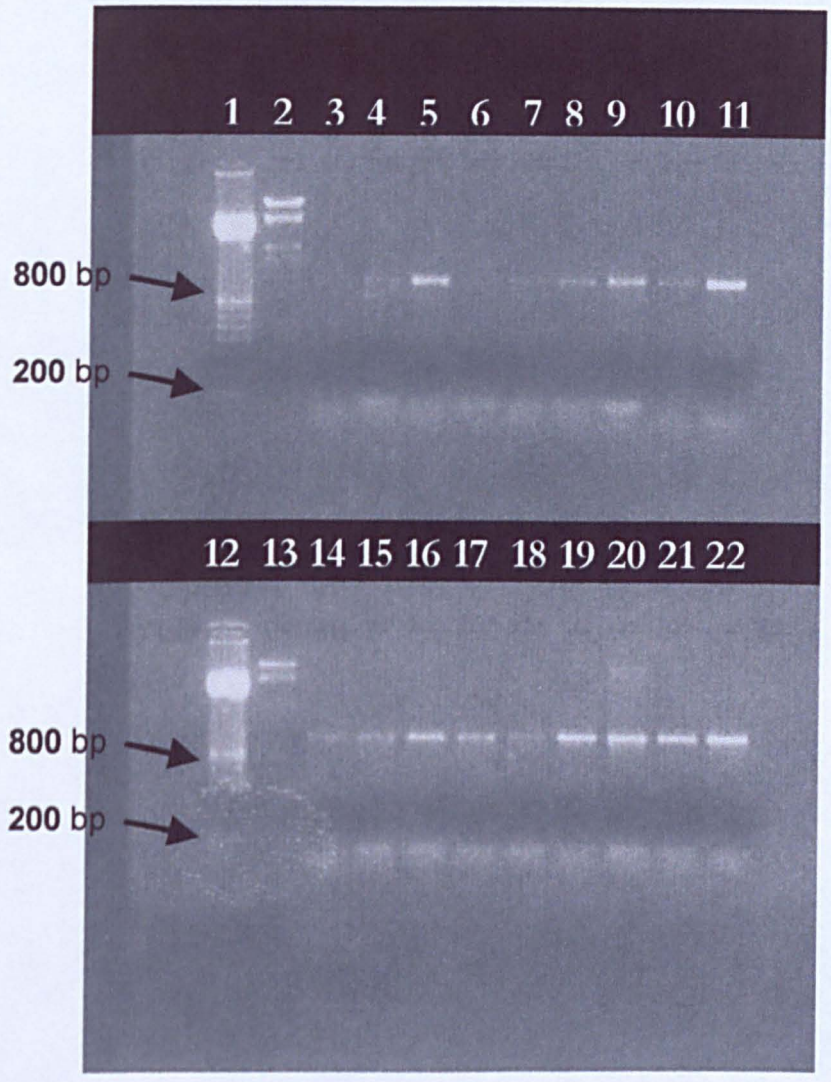
The standard ARDRA PCR conditions were producing multiple bands for some samples and not the single band (~1kbp) as expected. The effect of the addition of glycerol (which reduces secondary structures in G:C rich DNA) and touchdown PCR were investigated. With or without glycerol the standard ARDRA PCR conditions produced multiple bands (top gel). Touchdown PCR achieved the production of the expected ~1kbp band with or without glycerol (bottom gel).



Lanes

1	100bp ladder
2	*Ps.putida* Lab strain
3-8	Soil isolates
9-11	Water isolates
12-15	Raw milk isolates

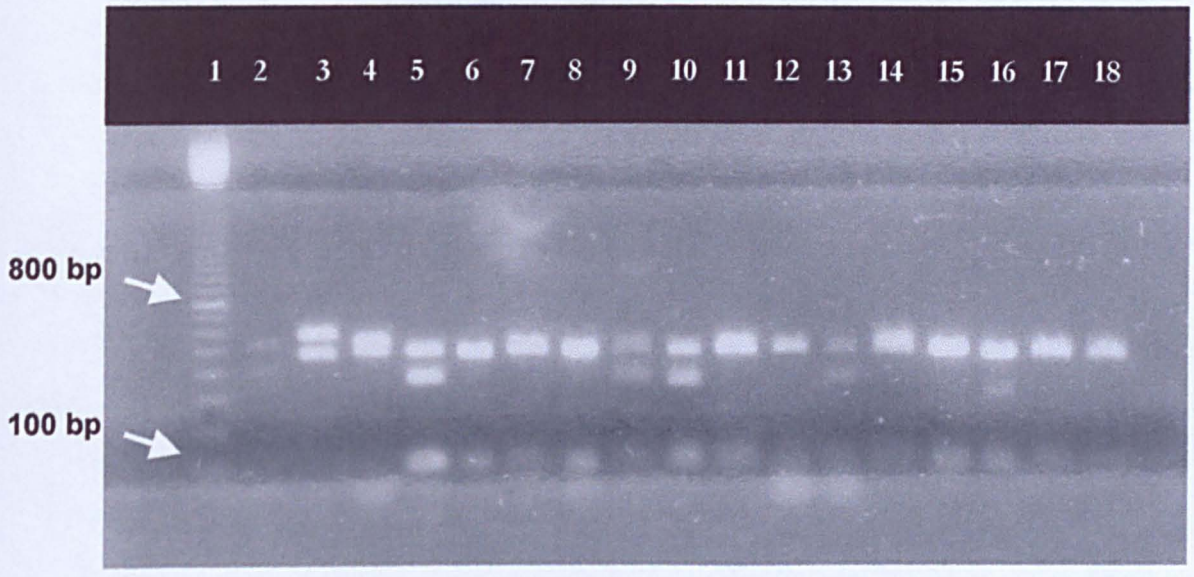
Figure 4.7 ARDRA PCR products from environmental pseudomonads using touchdown PCR protocol.



Lane		Lane	
1	100 bp λ ladder	12	100 bp λ ladder
2	λ *Eco*RI / *Hind* III marker	13	λ *Eco*RI / *Hind* III marker
3	*Ps. putida* - lab strain	14	*Ps. putida* - EM 11
4	*Ps. fluorescens* - NCTC 10038	15	*Ps. putida* - EM 12
5	*Ps. fluorescens* - EM 36	16	*Ps. putida* - EM 14
6	*Ps. aeruginosa* - NCTC 10332	17	*Ps. putida* - EM 17
7	*Ps. aeruginosa* - NO31	18	*Ps. putida* - EM 20
8	*Ps. alcaligenes* - NCTC 10367	19	*Ps. putida* Environmental-5
9	*Ps. alcaligenes* - PS 537	20	*Ps. putida* Environmental-6
10	*E. coli* - FSAC EJ1a	21	*Ps. putida* Environmental-8
11	*A. junii* - NCTC 12153	22	*Ps. fluorescens* Milk Isolate-36

Figure 4.8 ARDRA PCR products

The ARDRA PCR products were run (70V for 3 hours)on a 1.5 % agarose gel containing ethidium bromide. The arrow indicates the bright 800 base pair band.



LANE

1. 100 bp ladder
2. *Flavobacterium breve* NCTC 11099
3. *Acinetobacter junii* NCTC 12153
4. *Alcaligenes faecalis* NCTC 11953
5. *Shewanella putrefaciens* NCTC 10736
6. *Ps. acidovorans* NCTC 10683
7. *Ps. reptilovora* (old name) IFR 461
8. *Ps. fluorescens* NCTC10038
9. *Ps. aeruginosa* NCIMB 10545
10. *Ps. aeruginosa* NCTC 10332
11. *Ps. fluorescens* RM5
12. *Ps. fluorescens* RM1
13. *Ps. aeruginosa* NO31
14. *Ps. aeruginosa* EM31
15. *Ps. putida* EM20
16. *Ps. putida* EM17
17. *Ps. putida* EM14
18. *Ps. fluorescens* EM36
19. *Ps. testosteroni* NCTC10698

Figure 4.9 ARDRA PCR products digested with *Msp*I

Running conditions as for Figure 4.8

On subsequent repetitions of gene amplification the same results were not seen. Some isolates that had produced a single band now produced no band at all and some of those isolates that had produced multiples now produced a single band. The reproducibility of the assay was thus very poor.

Modifications to the ARDRA protocol were made to improve the reproducibility of the test and attain the desired target gene product. The unpredictability seen in the gene products produced suggested that a problem might have existed with the initial primer binding to the rDNA. Spurious binding of the primer to the DNA template would lead to the production of variously sized DNA fragments after amplification by PCR. The formation of multiple bands could also be due to there being too many cycles within the PCR protocol or that the annealling temperature was too low. Glycerol (5%) was added to the PCR reaction mixture to counter the possible formation of secondary structure within the DNA. In addition Touchdown PCR (Don *et al.*, 1991) was employed to overcome false priming during gene amplification.

The results of the optimisation of the ARDRA PCR conditions can be seen in Figure 4.6. With or without the addition of glycerol multiple bands were formed after gene amplification (Figure 4.6, top gel). Touchdown PCR produced the expected single band after gene amplification in the presence or absence of glycerol. Environmental *Pseudomonas* isolates from various sources (56 isolates tested) reproducibly produced the target band using the touchdown PCR protocol and a typical gel is shown in Figure 4.7.

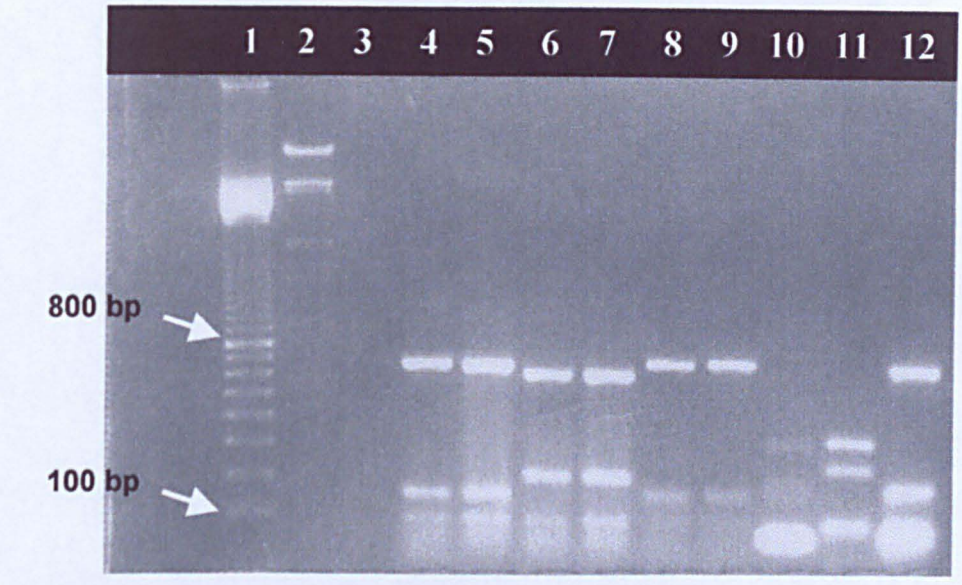
On occasion no gene amplification product would appear (Lane 3 & 6, Figure 4.8). The DNA for the PCR reaction is obtained directly from a colony and the amount present in the reaction was not quantified. If the amount of DNA used were too high then little or no product was seen and the same is true if the amount of DNA were too low.

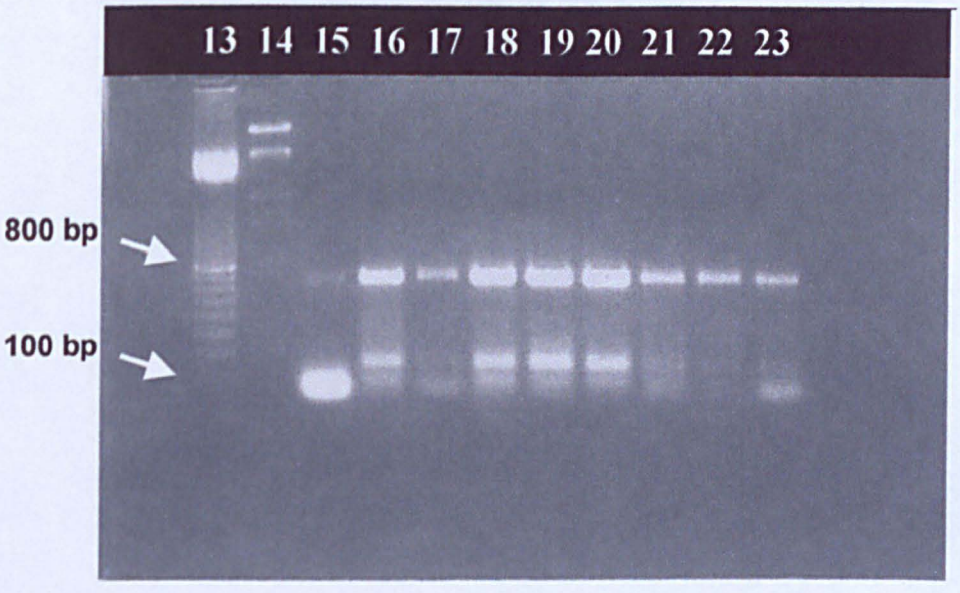
Restriction of amplified 16S rDNA was evaluated using *Msp*I (restriction site C↓CGG) and *Hae*III (restriction site GG↓CC). The cutting enzymes were chosen because the restriction sites contained guanidine and cytosine only. Fifty-six food and environmental isolates and standard strains were assayed with *Msp*I and twenty-two with *Hae*III. Figure 4.9 shows the products of the digests with *Msp*I. The banding pattern produced consisted of one or two bands of between 400 and 600 base pairs in size. All *Pseudomonas* food and environmental isolates produced one band of approximately 500 bp with the exception of most *Ps. aeruginosa* (6/7), some *Ps. putida* (3/10) and 1 *Pseudomonas* spp. that produced two bands.

Whilst *Flavobacterium*, *Acinetobacter* and *Alcaligenes* could be distinguished from *Pseudomonas* the pattern produced by *Shewanella putrefaciens* was identical to that produced by *Ps. aeruginosa*. The banding patterns produced thus had little discriminatory power between related genera. Within the *Pseudomonas* genus, only *Ps. aeruginosa* could be distinguished from other species. *Msp*I was thus of little value for confirmation of species types.

The ARDRA protocol using the *Msp*I restriction enzyme did not produce the desired outcome therefore an alternative enzyme (*Hae*III) was employed. Those isolates used to evaluate ribotyping were again used for subsequent ARDRA analyses to allow comparisons to be made between the two procedures.

The outcomes of the restriction digests with *Hae*III can be seen in Figure 4.10 and are summarised in Table 4.5. All genera tested including *Escherichia*, *Acinetobacter*, *Flavobacterium* (data not shown) and *Shewanella* (data not shown) produced distinct patterns. All *Pseudomonas* species tested (except *Ps. putida*) produced a unique banding pattern when the 16S rDNA PCR fragment was digested with *Hae*III. The *Ps. putida* isolates analysed fell into two groups, those with two bands (EM11, EM14, EM17 & EM20) and those with one (*Ps. putida* lab strain, EM12, Environmental-5 & Environmental-6). As previously stated *Ps.putida* is known to have two biovars (Stanier *et al.*, 1966). The strain Environmental-8 (*Ps. putida*), produced a single band that was of a different molecular weight to that of the other *Ps.putida* isolates that produced one band suggesting it may not be of the same species. For the eight *Ps. putida* strains tested (excluding Environmental-8) ribotyping produced five banding patterns and ARDRA produced two. ARDRA placed EM17, EM20, EM11 and EM14 into one group. Ribotyping sub-speciated the isolates of this group to form two sub-groups and grouped together EM17 with EM20 and EM11 with EM14, all other *Ps.putida* isolates had unique patterns.





Lane		Lane	
1	100 bp λ ladder	13	100 bp λ ladder
2	λ *Eco*RI / *Hind*III marker	14	λ *Eco*RI / *Hind*III marker
3	*Ps. putida* - lab strain	15	*Ps. putida* - lab strain
4	*Ps. fluorescens* - NCTC 10038	16	*Ps. putida* - EM 11
5	*Ps. fluorescens* - EM 36	17	*Ps. putida* - EM 12
6	*Ps. aeruginosa* - NCTC 10332	18	*Ps. putida* - EM 14
7	*Ps. aeruginosa* - NO31	19	*Ps. putida* - EM 17
8	*Ps. alcaligenes* - NCTC 10367	20	*Ps. putida* - EM 20
9	*Ps. alcaligenes* - PS 537	21	*Ps. putida* Environmental- 5
10	*E. coli* - FSAC EJ1a	22	*Ps. putida* Environmental - 6
11	*A. junii* - NCTC 12153	23	*Ps. putida* Environmental - 8
12	*Ps. fluorescens* Milk Isolate-36		

Figure 4.10 ARDRA PCR products digested with *Hae*III

Running conditions are as for Figure 4.8

ORGANISM	DISTANCE TRAVELLED BY FRAGMENTS (mm)		
	1	2	3
P. putida - lab strain	31		
P. fluorescens - NCTC 10038	35	51	54
P. fluorescens - EM 36	35	51	54
P. aeruginosa - NCTC 10332	36	49	54
P. aeruginosa - NO31	36	49	54
P. alcaligenes - NCTC 10367	35	52	54
P. alcaligenes - PS 537	35	52	54
E. coli - FSAC EJ1a	46	49	54
A. junii - NCTC 12153	45	48	56
Ps. fluorescens (milk isolate-36)	35	51	54
P. putida - EM11	31	44	
P. putida - EM 12	31		
P. putida - EM 14	31	44	
P. putida - EM 17	31	44	
P. putida - EM 20	31	44	
Ps. putida Environmental- 5	31		
Ps. putida Environmental - 6	31		
Ps. putida Environmental - 8	32		

Table 4.5 Distance travelled by each ARDRA product digested with *Hae*III

The ARDRA protocol developed using *Hae*III was sufficiently discriminatory, reproducible and rapid to allow it to be used for identification. The banding patterns produced by *Hae*III supported the identifications given by the API 20NE system for the isolates tested and potentially could be used for those not given species designations by API 20NE. As with ribotyping the choice of restriction enzymes was critical to the success of the assay.

The speed of the protocol was enhanced because the analysis could be conducted straight from the colony without the need to extract genomic DNA. One disadvantage to the procedure was that the ARDRA product was not produced first time with several strains and two or three attempts had to be made. This was improved by the use of touchdown PCR. The marriage of touchdown PCR with ARDRA has not to date been reported in the literature.

CHAPTER 5

NUMERICAL TAXONOMY

5.1 INTRODUCTION

Identification of the isolated environmental and food spoilage *Pseudomonas* organisms (Chapter 4) was achieved using classical biochemical tests and the API 20 NE system. Many isolates, however, were not identified beyond the genus level. The categorisation of some known strains was inaccurately described using API 20 NE and the application of classical biochemical tests at times produced inconclusive results with unknown isolates.

To elucidate the relationship of unknown isolates with each other and with standard strains, numerical analysis was performed on each biochemical profile. Due to the heterogeneity and the lack of reactivity of the *Pseudomonas* genus in many commonly used tests to differentiate between the Enterobacteriaceae, a large numbers of tests are required to differentiate group members (Costas *et al.*, 1992). Consequently, the Biolog system (Biolog Inc., Hayward, CA, USA), which is based on a 96-well microtitre tray containing 95 dehydrated carbon sources for assimilation and oxidation tests (Bochner, 1989), was chosen as the format to generate profile data.

The Biolog GN Microplate™ system is semi-automated and can be linked to the manufacturer's database to attain microbial identification. The use of the Biolog system for identification of bacteria has been given mixed endorsements of reliability (Miller and Rhoden, 1991; Costas *et al.*, 1992; Jones *et al.*, 1993; Miller *et al.*, 1993). In this study, however, the emphasis was placed on the relatedness and not the naming of the unknown organisms. To this end, the Biolog GN Microplate™ assay was used to process a large number of biochemical tests in a rapid and standardised format.

5.1.1 NUMERICAL TAXONOMY

Numerical taxonomy is defined as "*the grouping by numerical methods of taxonomic units into taxa based on their character states*" (Sneath and Sokal, 1973).

The application of numerical taxonomy to bacteria was introduced by Sneath (1957a; b) and the stages involved are summarised in Figure 5.1. An essential feature of this methodology is that initially, all the characters have equal importance or weight. Classically, taxonomists require that some tests are more important than others for defining taxa and that these should be used to establish the classification. Numerical taxonomists state that all characters should be of equal weight in the construction of classifications. Differential characters may be weighted once the taxa are defined.

An operational taxonomic unit (OTU) is the term used for each organism that is being studied (Sneath, 1972; Dunn and Everitt, 1982; Priest and Austin, 1993) and each of the range of tests performed on the OTUs are referred to as characteristics (Priest and Austin, 1993). Terminologies such as 'group and cluster', 'taxon, phenon and class' are used interchangeably by numerical taxonomists (Sackin and Jones, 1993).

The classifications obtained by numerical taxonomy should be objective and stable, unlike the subjective approach of a traditional taxonomist (Dunn and Everitt, 1982). However, the results of numerical taxonomy can be influenced by the statistics used, the presence of any test error and by any variations in the growth rates of the OTUs (Jones and Sackin, 1980; Goodfellow and Williams 1986). This means that the bacterial groupings may not be identical in different studies using alternative analysis methods (Jones and Sackin, 1980).

5.2 STAGES IN THE NUMERICAL ANALYSIS OF A BACTERIAL GROUP

5.2.1 STRAIN SELECTION

As a pre-requisite for numerical analyses, each OTU chosen needs to be assessed for purity (Priest and Austin, 1993). The study also necessitates the inclusion of reference strains

Figure 5.1 The Stages In Numerical Taxonomy Analysis Of Bacteria (based on Priest and Austin, 1993)

1. STRAIN SELECTION
    - need for pure cultures
    - inclusion of replicates
    - inclusion of reference cultures

2. TEST SELECTION
    - variety of tests
    - minimum of fifty tests
    - adoption of rapid methods where possible

3. RECORDING OF RESULTS
    - analysis of tests error and rejection of poorly reproducible tests

4. DATA CODING

5. COMPUTER ANALYSIS
    - calculation of similarities
    - cluster analysis

5. INTERPRETATION OF RESULTS
    - definition of clusters
    - identification scheme
    - selection of representative strains for allied studies

(i.e. culture collection strains) which act as marker strains (Jones and Sackin, 1980; Holmberg and Nord, 1984; Priest and Austin, 1993; Sackin and Jones, 1993).

With the aid of the available computer software over 600 OTUs can be handled as a single set; larger data sets may have to be divided. A minimum number of 60 OTUs is required to justify the use of computer-based analysis. With large data sets it may be prohibitively expensive or time consuming to duplicate all the results attained. However approximately ten per cent of the chosen OTUs should be duplicated to enable an estimate of the experimental test error to be calculated (Priest and Austin, 1993; Thompson, 1996).

5.2.2 TEST (CHARACTER) SELECTION

When conducting a numerical taxonomic study with bacteria any of the usual morphological, biochemical, serological or genetic characters used in classical studies can be incorporated. Any characters that are unable to distinguish between the OTUs (called redundant tests) need to be removed from the analysis, along with any non-reproducible tests (Priest and Austin, 1993; Logan, 1994).

A minimum of 50 (Jones and Sackin, 1980; Dunn and Everitt, 1982; Priest and Austin, 1993) and an optimum of between 100-150 characters should provide a stable classification. The characters chosen should be independent of each other (Bascomb, 1989) and preferably not be influenced by environmental changes (Priest and Austin, 1993). The tests used must be highly standardised to facilitate reproducibility and hence the stability of the classification. This requirement has influenced the utilisation of commercially available identification kits to provide the data for a number of numerical taxonomy studies (Thompson, 1996) because these kits are rapid, standardised and simple to use (Priest and Austin, 1993).

5.2.3 RECORDING RESULTS (TEST ERROR)

The reproducibility of a test can be influenced by any aspect of the testing procedure including the inoculum size, incubation temperature and media components (O'Brien and Colwell, 1987). Sneath and Johnson (1972) highlighted the possibility that erroneous test results would affect the numerical analysis. Sneath and Johnson (1972) suggested that if an individual test has a probability of error greater than 0.1-0.15 (i.e. 10-15%) it should be discarded from the analysis, although they also stated that a limited number of non-reproducible tests are acceptable. A large number of characters should decrease the effect that any non-reproducible tests have on the analysis (Priest and Austin, 1993).

5.2.4 CODING

Qualitative tests that give either a positive or negative result (i.e. binary or two state character) can be coded as 1 and 0 respectively (Sneath and Sokal, 1973; Sackin and Jones, 1993). Missing data can be scored as 1e9 or 9 (meaning no comparison) (Sneath, 1957b; Sokal and Sneath, 1963; Lockhart, 1970; Sneath and Sokal, 1973). This form of data is the most common in bacterial taxonomic studies (O'Brien and Colwell, 1987). Qualitative data can be scored additively (e.g. colour intensity) or as direct numerical values (e.g. colony size).

5.2.5 COMPUTER ANALYSIS

The incorporation of the data into computer packages such as NTSYSpc version 2.02f (F. James Rohlf, Exeter Software, New York) enables the level of similarity (distance) to be calculated between each OTU, followed by clustering analysis to order the OTUs into groups.

There are numerous equations, referred to as coefficients, which calculate the similarity or distance between data (Sneath and Sokal, 1973). The coefficients that are used mainly in numerical taxonomy studies of bacteria involving binary data are the simple matching coefficient (S_{SM})(similarity)(Sokal and Michener, 1958) and Manhattan distance (D). These coefficients are shown in Equations 5.1.

The S_{SM} coefficient, which ranges from 0 to 1 (identical) measures the number of matches as a proportion of the total number of characters, whereas the converse is true for Manhattan distance where 0 is identical. The S_{SM} and D^2 coefficient consider a negative match between two OTUs to be of equal importance to a positive match. However if the negative match relates to the inability of the organism to display the character, it has been suggested that other coefficients may be more appropriate i.e. Jacard coefficient (S_J; Dunn and Everitt, 1982). Sackin and Jones (1993) stated that the S_J coefficient should be used with slow growing organisms or other data that contain an excess of negative values. Pankhurst (1991) stated that negative matches should be included if the OTUs are closely related. The choice of coefficient used is therefore subjective (Pankhurst, 1991) and will determine the similarity values that are obtained (Priest and Austin, 1993).

Equations 5-1 Similarity Coefficients

Simple matching coefficient, $S_{SM} = (a + d) \div (a + b + c + d)$

Manhattan distance, $D^2 = 1 - S_{SM}$

Jacard coefficient, $S_J = a \div (a + b + c)$

Gower coefficient,

$$S_G = \frac{\left[1 - (\text{value of OTU1for a character} - \text{value of OTU2 for same character})\right]}{\text{range of values for the character}}$$

a and d = the number of positive and negative matches, respectively

b and c = the number of non-similar characters between the two OTUs.

Another similarity coefficient that has been used in numerical taxonomy studies involving bacteria (e.g. Logan *et al.*, 1979) is the similarity coefficient of Gower (S_G; Gower, 1971). The coefficient is a weighted average of all the similarity values between pairs of OTUs and can be used with a mixture of binary, quantitative and qualitative data (Sneath and Sokal, 1973; Priest and Austin, 1993). Unlike the S_{SM} and S_J coefficients, this coefficient allows for only partial similarity between OTUs (Jones and Sackin, 1980). When only two-state characters are compared the S_G coefficient is equivalent to the S_J coefficient (Sneath and Sokal, 1973).

5.2.6 CLUSTERING ANALYSIS

The OTUs are ordered into groups of high similarity using hierarchical, non-hierarchical (partitioning methods) or ordination methods (Sackin and Jones, 1993). Only the former will provide a ranked classification (Priest and Austin, 1993). The result obtained from clustering

procedures is a simplified relationship between the OTUs because mathematically each OTU belongs in a multidimensional attribute (A space) with the graph axes equalling the characters. The position of the OTU is based on the character value, but the picture becomes complicated because each character has its own axis, which will be at right angles to the other axes. The aim of the clustering technique is to simplify this multidimensional picture to two dimensions, but this obviously introduces some error into the result; however this is reduced at high similarity levels (Priest and Austin, 1993, Thompson, 1996). With hierarchical clustering the distances between close neighbours are accurately shown, however those between major groups are not. The reverse is seen with ordination methods, because these distort the distances between close neighbours (Sokal and Sneath, 1963; Logan, 1980; Logan and Berkeley, 1981; Alderson, 1985; Sackin and Jones, 1993; Logan, 1994). The former method therefore displays the taxonomic structure, whereas the latter indicates the taxonomic relationships (Sneath and Sokal, 1973; see Logan, 1980).

5.2.7 HIERARCHICAL CLUSTERING

There are a number of algorithms used for hierarchical clustering. However, single linkage (nearest neighbour) (Sneath, 1957b) and average-linkage, in particular the unweighted pair group method using arithmetic means (UPGMA) (Sokal and Michener, 1958), are the most popular (Priest and Austin, 1993). Both methods are linkage-based and so initially the highest similarity value between a pair of OTUs is chosen and these then group to become one OTU (Priest and Austin, 1993) forming a group or cluster (Jones and Sackin, 1980; Sackin and Jones, 1993). The pair group terminology arises because there are only ever two OTUs involved in forming a group (Boyce, 1969). The next highest similarity value is chosen either between two new OTUs or one new OTU and the group that has been formed, continuing until all of the OTUs are grouped (Jones and Sackin, 1980). The single linkage and UPGMA vary in the way that the OTUs join the clusters (Priest and Austin, 1993). Bascomb (1989) defined the two methods as follows: with single linkage "the distance between two clusters is that between their closest points" and with UPGMA "the distance between clusters equals the average distance between all pairs of cases". With single linkage therefore the similarity between groups is that seen between the two most similar OTUs between each group (Jones and Sackin, 1980) and has the effect of decreasing any differences between the clusters (Priest and Austin, 1993). The reverse of single linkage is complete linkage (furthest neighbour) (Sørensen, 1948) and this uses the two OTUs with the lowest similarity. With UPGMA, the average relates to the arithmetic average and so all OTUs are equally weighted (Jones and

Sackin, 1980). Weighting can be introduced by incorporating group size into the similarity calculation (Pankhurst, 1991).

A problem when using single linkage is that it exaggerates any similarity between groups (Pankhurst, 1991). The effect of the exaggeration of similarity means that distinct groups of OTUs can become linked at high similarity levels by a chain of OTUs, referred to as chaining. Chaining often occurs when intermediate OTUs are present, because these link together other clusters (Jardine and Sibson, 1968) and so obscure the presence of distinct groups (Dunn and Everitt, 1982; Sackin and Jones, 1993). Sokal and Sneath (1963) described these clusters as serpentine clusters and stated that the OTUs at the ends of such clusters do not have very much similarity.

Jones and Sackin (1980) suggested comparing UPGMA and single linkage dendrograms because straggly clusters can appear as sub-clusters on the UPGMA dendrogram. These can however be identified on the single linkage dendrogram because the entire cluster joins over a small similarity range (Jones and Sackin, 1980). However straggly clusters are less likely to occur when using binary data (Sackin and Jones, 1993). The comparison of UPGMA and single linkage algorithms therefore allows any OTUs which do not cluster effectively to be identified (Sackin, 1985). The use of average similarity values with the UPGMA method means that in theory this should be the most accurate procedure and provide the least distortion (Priest and Austin, 1993). However, the use of more than one method is suggested because clusters indicated by a number of different methods are more likely to be significant (Bascomb, 1989; Sackin and Jones, 1993).

5.2.7.1 Presentation of Hierarchical Clustering

There are a number of ways to display the hierarchical clustering results and these include:

1. *An ordered dissimilarity/similarity matrix* - in which the OTUs are listed according to the order of the clustering. The dissimilarity/similarity of each pair of OTUs is listed, providing a triangular array of data (Priest and Austin, 1993).

2. *A shaded dissimilarity/similarity matrix* - the ordered dissimilarity/similarity matrix is diagonally cut where 100% dissimilarity or similarity is seen, which occurs when the organism is compared with itself and above and below this diagonal the matrix is symmetrical (Sneath, 1962; Lessel and Holt, 1970). The similarity values are shaded and the higher the similarity, the darker the shading (Bascomb, 1989; Priest and Austin, 1993; Sackin and Jones, 1993). Triangular darkly shaded regions indicate the clusters. An advantage of this presentation is that OTUs that share similarity but belong to different clusters, can be seen by dark shading outside the areas relating to clusters (Priest and Austin, 1993).

3. *Dendrogram* - these are tree-like diagrams based on the phenetic similarities of the OTUs (Sokal and Rohlf, 1962; Sokal and Sneath, 1963). They are sometimes called phenograms to distinguish them from phylogenetic trees or cladograms (Sneath, 1972; Jones and Sackin, 1980). A single horizontal line represents each OTU. A vertical line at a particular level of similarity joins the OTUs within a cluster. A good cluster fuses all of the OTUs at a high similarity and the vertical stem is long with no other OTUs joining onto the group (Sackin and Jones, 1993). When the combination of the S_{SM} (or D^2) coefficient and the UPGMA algorithm are used the general boundaries for a species are at the 80-85% similarity level (or 15 - 20% dissimilarity) and a genus at the 60-65% similarity level (or 35-40% dissimilarity), however these values are not fixed (Priest and Austin, 1993).

   The accuracy of a dendrogram can be calculated using the cophenetic correlation coefficient (Sokal and Rohlf, 1962) which compares the original similarity coefficients with those (cophenetic values) obtained from the dendrogram (Sneath and Sokal, 1973; Sokal and Sneath, 1963). The comparison is achieved using the Pearson product-moment correlation coefficient (r) and the nearer the value to 1 the more similar the two matrices. Cophenetic correlation coefficient values are usually within the range of 0.6 to 0.95 and if greater than 0.8 they are described as 'reasonably good' (Sackin and Jones, 1993) or 'acceptable' (Pankhurst, 1991). If r is less than 0.7 then the classification is 'poor' and only of limited use (Jones and Sackin, 1980; Sackin and Jones, 1993).

5.2.8 Analysis of Hierarchical Clustering

There are a number of ways to analyse the hierarchical clustering data and these include:

1. Intra and inter group similarity values - the average similarity value within and between groups identifies clusters that contain very similar organisms and whether clusters are distinct (Priest and Austin, 1993).

2. Overlap between clusters - The concept of overlapping clusters was described by Jardine and Sibson (1968) and implies that, at a set similarity, an OTU may exist in more than one cluster (see Sackin and Jones, 1993). This procedure can identify intermediate OTUs (Sackin and Jones, 1993). Assessment of overlap is also of value before obtaining an identification matrix because the OTUs within any overlapping clusters might only be distinguished using further characters (Priest and Williams, 1993). The overlap is obtained between each pair of clusters and examined to see if it is lower than a set expected (critical) overlap value (Sneath, 1979; see Sackin, 1987). The calculation of overlap requires that the distance of each OTU from the centroid (the average organism: Sneath and Sokal, 1973) in Euclidean space (Sneath, 1979) be obtained. These distances are then projected onto their intercentroid axis and the index of disjunction (W), that corresponds to a degree of overlap (V), is calculated (Sneath, 1979). Some programs also utilise estimated intercentroid distances which account for the fact that the populations being examined are only a subset of a larger population. These intercentroid distances are generally shorter than the observed ones between the clusters. Hence use of these values is a more rigorous test of the degree of overlap (Thompson, 1996).

3. Percentage frequency of occurrence of each character in the taxon - this provides a frequency matrix that can act as a database for the identification of unknown organisms (Holmberg and Nord, 1984; Priest and Austin, 1993). This can only be achieved with binary data (Sackin and Jones, 1993). The data can form a diagnostic table containing all characters or just those of value (Holmberg and Nord, 1984; Priest and Austin, 1993) or the latter weighted characters can be incorporated into identification keys (Holmberg and Nord, 1984).

5.2.9 ORDINATION

Dendrograms and sorted similarity matrices are one or two-dimensional representations of a given data set. To attain such simplicity a lot of information has been lost. If the data could be looked at in space of several dimensions then much of this loss could be avoided. Multivariate analysis seeks to determine the best condensation of multidimensional relationships onto a reduced number of planes (Clifford and Stephenson, 1975). Multivariate methodology can be applied to one of four situations. The only one of relevance here is ordination. Ordination makes no assumptions about the existence of groupings among the OTUs and operates using either the attribute scores (Principal component analysis) or the dissimilarity matrix (Principal co-ordinate analysis).

Principal co-ordinate analysis plots the inter-OTU dissimilarities as distances into a set of orthogonal axes, such that the OTU values on the new axes preserves the original relationships. Ordination plots reflects accurately the relationships between groups but may be less accurate in representing the relationship between near neighbours (Sneath and Sokal, 1973).

5.3 DETERMINATIVE TEST METHODS

5.3.1 STRAINS

Eighty-four environmental *Pseudomonas* isolates were assayed together with eleven *Pseudomonas* reference strains and five non-*Pseudomonas* strains (Table 5.2). The non-*Pseudomonas* reference group included other Gram negative spoilage genera such as *Flavobacterium*, *Acinetobacter* and *Alcaligenes*. In this analysis 19 % of the OTUs were duplicated.

5.3.2 Biolog GN Microplate™

The Biolog GN MicroPlate™ has a panel of 95 different carbon sources plus a negative control well. The well-by-well listing can be seen in Figure 5.2.

5.3.2.1 Inoculum preparation

Freshly grown pure cultures of the test organism were inoculated twice onto TSA plates (18 h, 30°C) to assure purity, promote vigorous growth and retain metabolic activity. A uniform suspension was prepared by dipping a sterile swab into sterile saline and removing the cells from the surface of the plate by rolling the premoistened swab over the colonies. The swab was then pressed onto the inside surface of a bottle of warm sterile saline (30°C, 20ml) above the water line to break up clumps and to release cells into the saline. The solution was gently mixed using a sterile pipette to produce a uniform suspension. The inoculum density was adjusted to an OD of 0.3-0.35 at 600nm wavelength (Gallenkampf Visi-Spec).

5.3.2.2 Reading the Microplate™

The plates were read using an automatic plate reader (Dynatech 5000, 630nm). Each plate was shaken for 20 seconds before reading to dissipate the purple precipitate that may settle out at the bottom of the well during incubation.

A 1	Water	E 1	P- Hydroxyphenyl Acetic Acid
A 2	A- Cyclodextrin	E 2	Itaconic Acid
A 3	Dextrin	E 3	A- Keto-Butyric Acid
A 4	Glycogen	E 4	A- Keto-Glutaric Acid
A 5	Tween 40	E 5	A- Keto-Valeric Acid
A 6	Tween 80	E 6	D,L- Lactic Acid
A 7	N- Acetyl Galactosamine	E 7	Malonic Acid
A 8	N- Acetyl-D-Glucosamine	E 8	Propionic Acid
A 9	Adonitol	E 9	Quinic Acid
A 10	L- Arabinose	E 10	D- Saccharic Acid
A 11	D- Arabitol	E 11	Sebacic Acid
A 12	D- Cellobiose	E 12	Succinic Acid
B 1	I- Erythritol	F 1	Bromo Succinic Acid
B 2	D- Fructose	F 2	Succinamic Acid
B 3	L- Fucose	F 3	Glucuronamide
B 4	D- Galactose	F 4	Alaninamide
B 5	Gentiobiose	F 5	D- Alanine
B 6	A-D- Glucose	F 6	L- Alanine
B 7	M- Inositol	F 7	L- Alanyl-Glycine
B 8	A-D- Lactose	F 8	L- Asparagine
B 9	Lactulose	F 9	L- Aspartic Acid
B 10	Maltose	F 10	L- Glutamic Acid
B 11	D- Mannitol	F 11	Glycyl-L-Aspartic Acid
B 12	D- Mannose	F 12	Glycyl-L-Glutamic Acid
C 1	D- Melibiose	G 1	L- Histidine
C 2	B- Methyl-D-Glucoside	G 2	Hydroxy-L-Proline
C 3	D- Psicose	G 3	L- Leucine
C 4	D- Raffinose	G 4	L- Ornithine
C 5	L- Rhamnose	G 5	L- Phenylalanine
C 6	D- Sorbitol	G 6	L- Proline
C 7	Sucrose	G 7	L- Pyroglutamic Acid
C 8	D- Trehalose	G 8	D- Serine
C 9	Turanose	G 9	L- Serine
C 10	Xylitol	G 10	L- Threonine
C 11	Methyl Pyruvate	G 11	Carnitine
C 12	Methyl Succinate	G 12	G- Amino Butyric Acid
D 1	Acetic Acid	H 1	Urocanic Acid
D 2	Cis- Aconitic Acid	H 2	Inosine
D 3	Citric Acid	H 3	Uridine
D 4	Formic Acid	H 4	Thymidine
D 5	D- Galactonic Acid Lactone	H 5	Phenylethylamine
D 6	D- Galacturonic Acid	H 6	Putrescine
D 7	D- Gluconic Acid	H 7	2- Amino Ethanol
D 8	D- Glucosaminic Acid	H 8	2,3- Butanediol
D 9	D- Glucuronic Acid	H 9	Glycerol
D 10	A- Hydroxybutyric Acid	H 10	D,L-A- Glycerol Phosphate
D 11	B- Hydroxybutyric Acid	H 11	Glucose-1-Phosphate
D 12	G-Hydroxybutyric Acid	H12	Glucose-6-Phosphate

Figure 5.2 Well-by-well listing of the Biolog GN Microplate™ Reagents

5.3.2.3 Coding of results

A positive result was scored if the reading given was more than or equal to 140% of the value given by the blank cell and a negative result was scored if the value was less than 140% of the blank cell. This generated a binary data set.

A qualitative data set was generated with the same data by scoring each value as follows:

Value (≥)	Score
120% x Blank	1
140% x Blank	2
160% x Blank	3
180% x Blank	4
200% x Blank	5

The qualitative data set was defined to establish whether the strength of a particular reaction, on the Microplate™, would be a more defining character when determining relatedness than a simple positive or negative result. The aim was to see if a more accurate or stable relatedness matrix could be attained.

5.3.2.4 Experimental test error

Nineteen percent of the OTUs were examined in duplicate. Any test that generated variable results for the same OTU within the binary data set was scored with a 9. The qualitative data set was analysed without modification.

Each OTU that was duplicated generated two data sets, the second set was denoted by .1 being added to the OTU code number e.g. 1 and 1.1. All of the Biolog raw data is tabulated in the Appendix.

5.4 TAXOMETRIC METHOD

A number of computer programs were used to perform the numerical analyses and are shown in Table 5.1.

5.4.1 HIERARCHICAL CLUSTERING

The similarity coefficient used was Manhattan distance (D^2). The hierarchical clustering of the binary and qualitative data was achieved using the single linkage (SL), the UPGMA and the complete linkage (CL) algorithms. The Pearson product moment correlation coefficient was calculated for each dendrogram. The ordered percentage distance matrix and the

ordered shaded matrix was generated from the dendrogram using the D^2 / UPGMA algorithm.

5.4.2 ORDINATION

The output of Principle co-ordinate analysis (NTSys2.0f) was represented as 2 and 3D plots in the first two or three dimensions respectively.

5.4.3 ANALYSIS OF OVERLAP BETWEEN GROUPS

The OVERMAT program was used to assess the degree of overlap between the groups obtained using the D^2/UPGMA algorithm. The program analysed all pairs of groups and obtained their intercentroid distances and the standard deviations of these distances from their own centroid of projections of OTUs along the intercentroid axis (Sackin, 1987). The critical overlap (V_O) was set at 1% and the confidence intervals used were P=90, 95 and 99%.

5.4.4 PERCENT POSITIVE TESTS FOR EACH TAXON

The table for the percent positive tests for each taxon was generated using PHS8T03 computer program.

COMPUTER PROGRAMS	FUNCTION OF PROGRAM	SOURCE
PHS8T03	A basic program which converts a matrix of binary data into a matrix of percent positive states given a taxon membership list for each OTU	P.H.A. Sneath and M.J. Sackin, Leicester University
OVERMAT	A basic program which calculates the extent to which pairs of groups overlap in an identification matrix.	Sneath, 1979
OVCLUST	A basic program that produces a significance test for the distinctness of a pair of clusters in Euclidean space	Sneath, 1979
DIACHAR	A basic program which determines the most diagnostic properties of groups or taxa. The most diagnostic states are those which have the majority state in the characters with the highest diagnostic scores.	Sneath, 1980
NTSYS (VERSION 2.02f)	A windows program that is used to display structure in multivariate data e.g. dendrograms or principle co-ordinate plots.	F. James. Rohlf, Exeter Software, New York

Table 5.1 Computer programs used during Numerical Analyses

5.5 RESULTS

Figure 5.3 shows a Biolog GN Microplate™ that was inoculated with an environmental *Pseudomonas* isolate (code number 67), after 18 hours incubation at 30°C.

5.5.1 HIERARCHIAL CLUSTERING

5.5.1.1 Dendrograms

For each algorithm several different dendrograms were produced (e.g. 25 for D^2/single linkage and 8 for D^2/UPGMA), each being known as a matrix. Each matrix is a slightly different representation of the data and all are equally valid. The differences between matrices in these cases were very small, with a single pair of OTUs being reversed, hence the overall relatedness structure was unchanged. Typical dendrograms resulting from classifying the 93 OTUs' binary data using single and complete linkage are shown in the Appendix. The dendrograms generated with the qualitative data using single linkage, UPGMA and complete linkage are also shown in the Appendix.

The dendrograms produced using the UPGMA algorithm, for both the binary and qualitative data, had a cophenetic correlation coefficient (CCC) of 0.80 and therefore produced a stable dendrogram for both types of data. Single linkage, for both types of data, produced a high degree of "chaining " or "snaking" of the OTUs, leading to long straggly clusters and thus the elucidation of groups between closely related OTUs would be difficult to achieve. The low correlation coefficients show that the single linkage algorithm represent very poorly the relatedness within both the binary (CCC 0.65) and qualitative data (CCC 0.63) sets. The complete linkage technique also failed to produce stable relatedness matrices for the binary and qualitative data sets with CCC values of 0.60 and 0.70, respectively. Thus, only the dendrograms generated using the UPGMA algorithm were acceptable for interpretation.

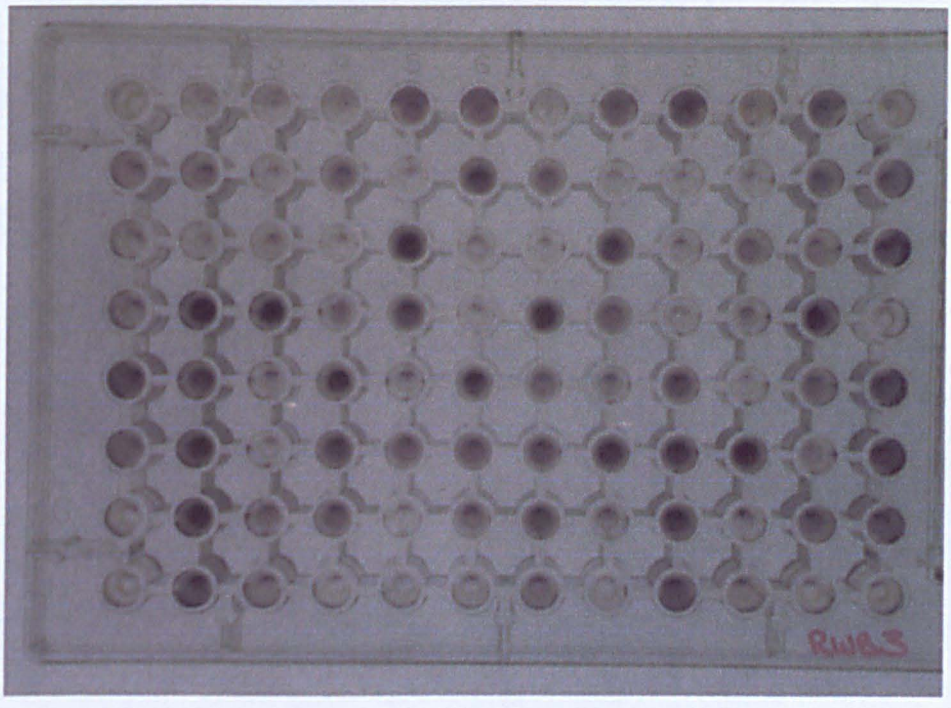


Figure 5.3 Biolog GN Microplate™ inoculated with an environmental *Pseudomonas* isolate

5.5.1.2 D^2/UPGMA algorithm

5.5.1.2.1 Qualitative data

The results for all of the OTUs and duplicates were used to generate the qualitative data set. No correction was made for any differences between replicate samples. The reason was that due to there being five categories that the data could fall into, there was a strong likelihood that the percentage variable results attained for each character would be high. If the number of variable results were above 15-20% (of 95 characters) then the validity of the relatedness matrix generated would be brought into question. The dendrogram produced using the D^2/UPGMA algorithm was "reasonably good" however; it was not more accurate than the dendrogram generated with the binary data with the same algorithm. Accordingly no further analysis of this data was done.

5.5.1.2.2 Binary data

The D^2/UPGMA dendrogram was divided into ten taxa at the 26% distance-level. (Figure 5.4). Each OTU present within each taxon is summarised in Table 5.2.

Those taxa that group at the 22% distance level or below (taxa 1, 2, 3, 4 and 5) are considered to be members of the same species. All strains that coalesce above this level may be considered presumptive members of a species. In general a generic boundary is denoted at the 35 % distance level (taxa 1-6; 7; 8-9; 10) All the OTUs coalesce at the 42% distance level indicating that all of the OTUs do not belong to one genus.

Taxon 1 tightly clusters at 19% distance. This taxon contains the *Ps. fluorescens* NCTC 10038 reference strain and 78% of the strains within the group were identified as *Ps. fluorescens*. Of the 32 members of this group four were not speciated. The organisms within this taxon originated from a range of environments e.g. raw and pasteurised milk, fish, river water and soil. Those strains isolated from the same source e.g. fish or raw milk tends to form sub-clusters (ecovars), grouping at distance levels as low as 7%.

Taxon 2 groups at the 18% distance level and contains no reference strains. Of the ten members of this group 70% were isolated from river water. The API 20NE presumptive

Table 5.2 Source and presumptive identification of the members of each taxon

CODE	PRESUMPTIVE ID (API)	SOURCE	TAXON
1	*Ps.fluorescens*	Fish	1
7	*Ps.fluorescens*	Fish	1
10	*Ps.putida*	Fish	1
50	*Ps.fluorescens*	Pondwater	1
70	*Ps.fluorescens*	Riverwater	1
91	*Ps.fluorescens*	NCTC 10038	1
2	*Ps.fluorescens*	Fish	1
6	*Ps.fluorescens*	Fish	1
12	*Ps.putida*	Fish	1
4	*Ps.fluorescens*	Fish	1
3	*Ps.fluorescens*	Fish	1
5	*Ps.fluorescens*	Fish	1
8	*Ps.fluorescens*	Fish	1
101	*Ps.putida*	Lab strain A	1
51	*Ps.fluorescens*	Pondwater	1
86	*Ps.fluorescens*	Soil	1
84	*Ps. chloroaphis*	Soil	1
9	*Ps.fluorescens*	Fish	1
47	*Pseudomonas spp*	Pondwater	1
54	*Ps.fluorescens*	Gutterwater	1
55	*Ps.fluorescens*	Gutterwater	1
53	*Ps.fluorescens*	Pondwater	1
28	*Ps.fluorescens*	Rawmilk	1
32	*Ps.fluorescens*	Rawmilk	1
30	*Ps.fluorescens*	Rawmilk	1
29	*Ps.fluorescens*	Rawmilk	1
56	*Pseudomonas spp*	Gutterwater	1
34	*Ps.fluorescens*	Past.milk	1
36	*Ps.fluorescens*	Past.milk	1
38	*Pseudomonas spp*	Past.milk	1
39	*Ps.fluorescens*	Past.milk	1
71	*Ps.fluorescens*	Riverwater	1
41	*Ps.aeruginosa*	Pork	2
15	*Ps.aeruginosa*	Fish	2
74	*Ps.fluorescens*	Riverwater	2
65	*Pseudomonas spp*	Riverwater	2
52	*Pseudomonas spp*	Pondwater	2
72	*Ps.fluorescens*	Riverwater	2
69	*Ps.fluorescens*	Riverwater	2
73	*Ps. pseudomallei*	Riverwater	2
42	*Ps.aeruginosa*	Pork	2
66	*Pseudomonas spp*	Riverwater	2
33	*Ps.fluorescens*	Past.milk	3
90	*Ps.aeruginosa*	NCIMB10548	3
68	*Pseudomonas spp*	Riverwater	3

Table 5.2 Source and presumptive identification of the members of each taxon

CODE	PRESUMPTIVE ID (API)	SOURCE	TAXON
11	*Ps.putida*	Fish	4
43	*Ps.putida*	Pork	4
13	*Ps.putida*	Fish	4
14	*Ps.putida*	Fish	4
19	*Ps.fluorescens*	Rawmilk	4
20	*Pseudomonas spp*	Rawmilk	4
31	*Pseudomonas spp*	Rawmilk	4
44	*Ps.fluorescens*	Pork	4
46	*Ps.putida*	Pork	4
45	*Ps.putida*	Pork	4
21	*Ps. picketti*	Rawmilk	4
25	*Ps.fluorescens*	Rawmilk	4
27	*Pseudomonas spp*	Rawmilk	4
23	*Ps.putida*	Rawmilk	4
24	*Ps.putida*	Rawmilk	4
22	*Ps.fluorescens*	Rawmilk	4
26	*Pseudomonas spp*	Rawmilk	4
17	*Ps.aeruginosa*	Chicken	5
88	*Ps.aeruginosa*	NCTC 10332	5
75	*Ps. mendocina*	Soil	5
87	*Ps.aeruginosa*	Soil	5
77	*Ps.aeruginosa*	Soil	5
57	*Pseudomonas spp*	Gutterwater	5
80	*Ps.chloroaphis/fluorescens*	Soil	5
79	*Ps.cepacia*	Soil	5
85	*Ps.fluorescens*	Soil	5
82	*Ps. chloroaphis*	Soil	5
94	*Ps.reptilovora*	IFR 461	5
76	*Pseudomonas spp*	Soil	5
83	*Ps.fluorescens*	Soil	5
103	*Ps.putida*	Lab strain C	5
67	*Pseudomonas spp*	Riverwater	6
59	*Ps.fluorescens*	Gutterwater	7
78	*Ps.fluorescens*	Soil	7
61	*Chryseomonas luteus*	Riverwater	7
99	*E.coli*	Lab strain Ej1a	7
48	*Ochrobacter anthropi*	Pondwater	8
96	*Shewanella putrefaciens*	NCTC 10736	8
16	*Ps.picketti*	Fish	8
93	*Ps.alcaligenes*	NCTC 10367	8
102	*Ps.putida*	Lab strain B	8
89	*Ps.putida*	Lab strain 3288	8
98	*Acinetobacter junii*	NCTC 12153	8
95	*Ps.acidovorans*	NCTC 10683	8
92	*Ps.testosteroni*	NCTC 10698	8
97	*Alcaligenes faecalis*	NCTC 11953	8
58	*Xanthomonas maltophilia*	Gutterwater	9
100	*Flavobacterium breve*	NCTC 11099	10

identification denoted 30% of the OTUs to be *Ps. aeruginosa*, 30% as *Ps. fluorescens*, 30 % as *Pseudomonas* species and 10% as *Ps. pseudomallei.*

Taxon 3 contained the *Ps. aeruginosa* NCIMB 10548 reference strain plus 2 other isolates, which were named by API 20 NE as *Ps. fluorescens* and a *Pseudomonas* species. This group clustered at the 22% distance level.

Taxon 4 did not contain a reference strain but had 47% of the isolates identified by API 20 NE as *Ps. putida* and 23 % were identified to the level of genus only. The group tied at the 22% distance level. The taxon could be further sub-divided into 2 groups. The source of the strains was a strong influence on the formation of sub-clusters e.g. raw milk. All of the organisms within the cluster originated from highly proteinaceous sources e.g. pork, raw milk and fish.

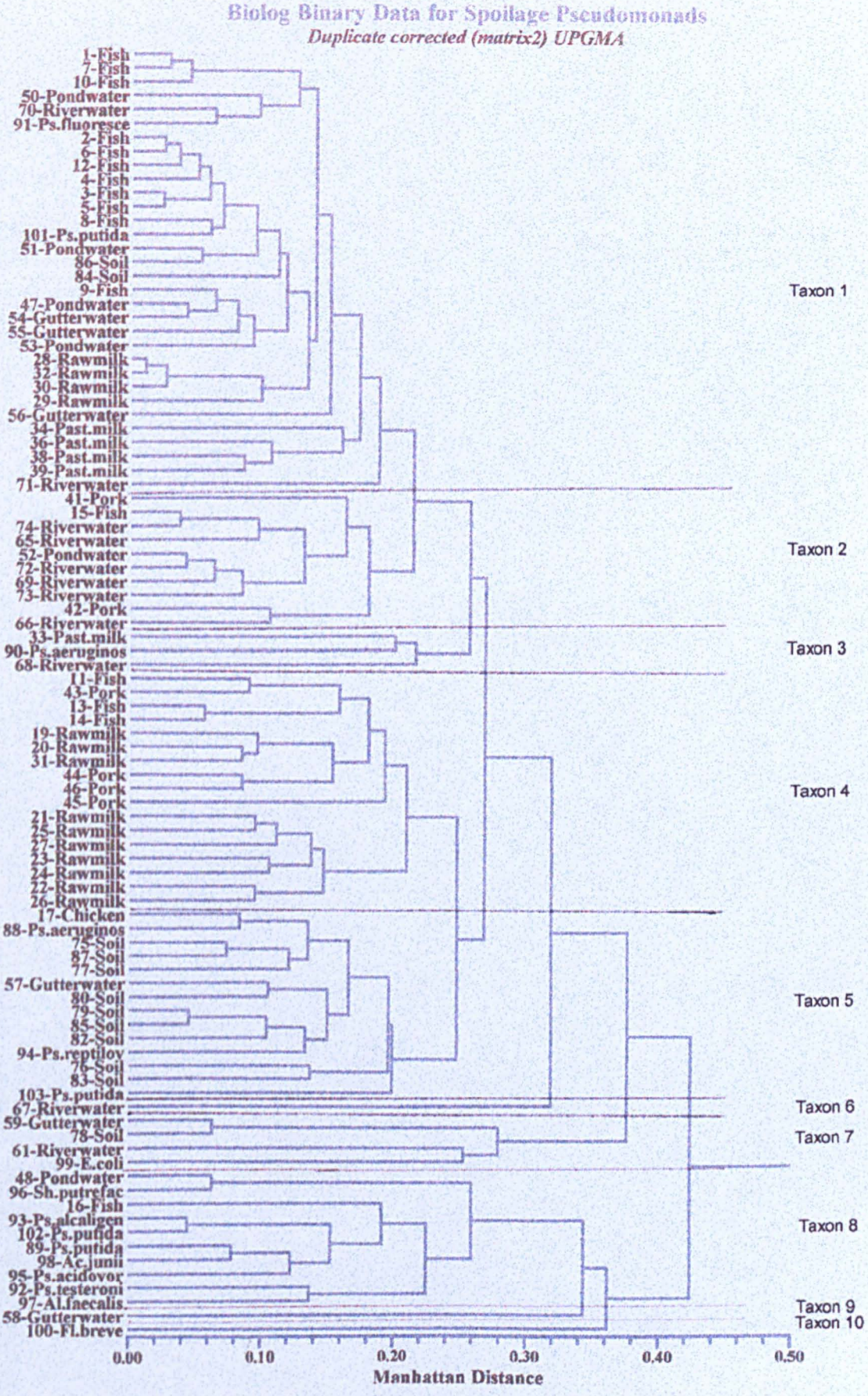
Taxon 5 grouped at the 20% distance level and contains the *Ps. aeruginosa* NCTC 10332 type strain and the *Ps. reptilovora* IFR 461 marker strain. Within this taxon there is an eclectic assortment of species (presumptively identified by API 20 NE) mainly sourced from the soil. These include *Ps. mendocina*, *Ps. fluorescens*, *Ps. putida*, *Ps. cepacia*, *Ps. chloroaphis*, *Ps. reptilovora* and *Ps. aeruginosa*. Of the 14 members of this group 28 % were classified as *Ps.aeruginosa* by API 20 NE. The taxon could be further sub-divided into at least two distinct sub-groups, one of which would contain the *Ps. aeruginosa* type strain and the other the *Ps. reptilovora* strain.

Taxon 6 was a singly occupied cluster (unidentified *Pseudomonas* river water isolate) as were taxa 9 (*Xanthomonas maltophila* from gutter water) and 10 (*Flavobacterium breve* NCTC 11099).

Taxon 7 grouped at the 28% distance level. Of the four members of this group 50% were presumptively identified as *Ps. fluorescens*. The group also contained the marker strain *E.coli* EJ1A and an isolate presumed to be *Chryseomonas luteus*. The two *Ps. fluorescens* species are closely related to each other and form a sub-group that ties at the 7% distance level.

Taxon 8 clusters at the 26% distance level, and is a very disparate group because it may contain five different genera including three of the five non-*Pseudomonas* reference strains and 5 *Pseudomonas* marker strains.

Figure 5.4 Dendrogram generated using the D^2/UPGMA algorithm



5.5.2 ORDERED DISTANCE MATRICES

An ordered shaded distance matrix (OSDM, Figure 5.5) was derived using the D^2/UPGMA algorithm. The OSDM visually represents taxa and sub-groups within taxon by triangle-shaped shading of low distance (or high similarity) on the hypotenuse of the matrix.

The members of taxon 1 form a highly cohesive group containing several discrete and overlapping sub-groups. For instance, OTUs 1, 7, 10, 50, 70, 91, 2, 6, 12, 4 and 3 (mainly originating from fish) form a discrete sub-group which overlaps with a larger subcluster containing OTUs 91, 2, 6, 12, 4, 3, 5, 8, 101, 51, 86, 84 and 9 (originating from fish, water and soil). Highly related (0-13% distance) discrete sub-groups include OTUs 9, 47, 54, 55 and 53 which originate from environmental waters and fish; OTUs 28, 32, 30 and 29 which all originate from raw milk; and OTUs 1, 7 and 10 which originate from fish.

Taxon 2 could be further split into three sub-clusters such that OTUs 41 and 15 would be grouped together, OTUs 74, 65, 52, 72, 69 and 73 would form another sub-group and OTUs 73 and 42 would form the final sub-group. Taxon 1 and taxon 2 can be seen to overlap, such that members of taxon 2 are related, at the 13-25% level, to members of taxon 1.

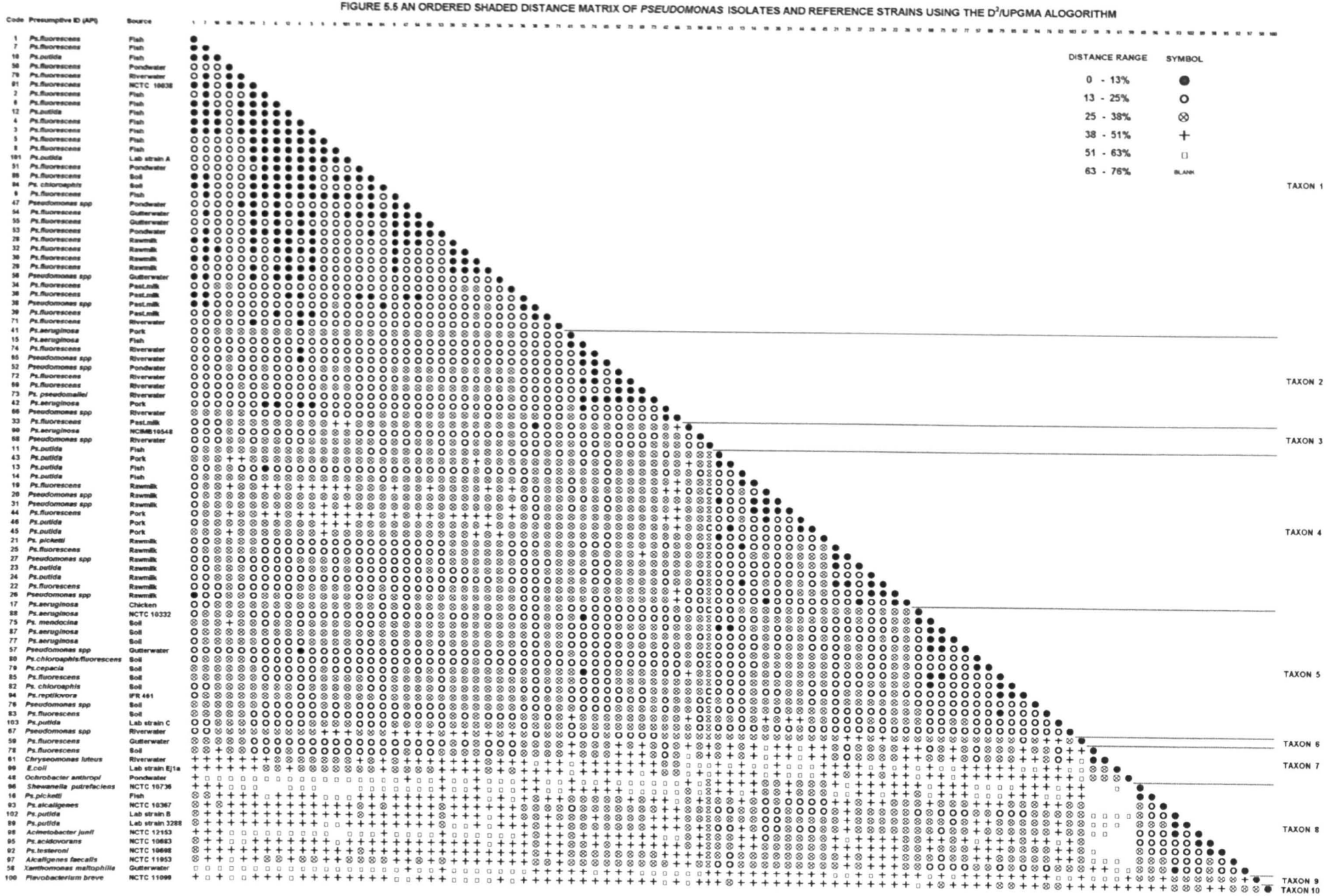
The three members of taxon 3 are related at the 13-25% level. Taxon 4 is not as cohesive a group as taxon 1 and contains a highly related sub-group that contains OTUs (24, 21, 27, 23, 24, 22 and 26) that originated from raw milk

Two highly related sub-groups can be seen in taxon 5.each containing three OTUs and each sub-group members originating from soil.

The two *Ps. fluorescens* isolates in taxon 7 are highly related to each other and they are distantly related to the other members of the taxon, which belong to different genera. The *Ps. fluorescens* isolates are shown to be related (13-25%) to several members of taxon 1.

Taxon 8 is a fairly disparate group with several pairs of isolates being highly related to each other. For instance, reference strains *Ps. putida* Lab strain B and *Ps. alcaligenes* are highly related as are *Acinetobacter junii* and *Ps. putida* Lab strain 3288.

FIGURE 5.5 AN ORDERED SHADED DISTANCE MATRIX OF *PSEUDOMONAS* ISOLATES AND REFERENCE STRAINS USING THE D^2/UPGMA ALOGORITHM



5.5.3 ANALYSIS OF OVERLAP

If any of the two taxa being compared contained too few OTUs (three or less), or differed greatly in OTU number or standard deviation, then the T statistic was not considered accurate. The valid results used in the interpretation of overlap of the taxa are shown in Table 5.3.

All pairs of taxa that were compared did not overlap by less than 1% at the 99% confidence level. The two exceptions to this were the comparison between taxon 4 & 7 and taxon 5 & 7. Each of these pairs of taxa overlapped by less than 1% at the 95% but not at the 99% confidence level. This indicates that an OTU from either taxon could co-exist on or near the boundaries between both groups. The degree of overlap seen however, was very small.

5.5.4 PERCENTAGE POSITVE CHARACTERS PER TAXON

The percentages of OTUs within each D^2/UPGMA taxon that were positive for each character (variable results were ignored) are shown in Figure 5.6. From this data the DIACHAR computer program generated a list of ten characters that were most diagnostic for each taxon (Table 5.4)

5.5.5 ORDINATION

The binary phenotypic data was analysed using Principle co-ordinate analysis and a two-dimensional representation is shown in Figures 5.7A and 5.7B (the axes represents the numbered OTUs co-ordinates in the first two dimensions). Groupings between the OTUs become more obvious when the members of the same taxon are colour coded as seen in Figure 5.7B. Taxon 1 appears as a tightly grouped cluster, which borders closely to taxon 2. Taxa 2, 3 4 and 5 appear to be less tightly grouped than taxon 1. Taxon 7 is a highly dispersed group whose members seem to be poorly related. Taxon 8 is also a highly dispersed group whose members could be further divided into sub-groups. All of the OTUs distributed on the left-hand side of the plot are non-pseudomonads. The OTUs distributed on the right-hand

Table 5.3 The Analysis of the Taxa groupings using OVERMAT

TAXON GROUP	T (W)	T (0, P = 0. 90)	T (0, P = 0. 95)	T (0, P = 0. 99)	OVERLAP SIGNIFICANCE
1 & 2	26.8	14.4	15.7	18.8	★★★★
1 & 5	33.9	14.2	15.2	17.4	★★★★
1 & 4	29.5	14.3	15.2	17.1	★★★★
1 & 8	43.7	14.7	16.2	19.6	★★★★
2 & 7	23.3	11.5	13.8	20.1	★★★★
2 & 8	33.6	9.9	10.9	12.8	★★★★
3 & 7	20.6	8.4	10.2	15.1	★★★★
4 & 5	25.1	11.6	12.4	19.5	★★★★
4 & 7	24.9	14.5	17.7	26.0	★★★
4 & 8	29.3	11.4	12.4	14.6	★★★★
5 & 7	23.8	13.4	16.2	23.9	★★★
5 & 8	31.2	10.8	11.7	13.8	★★★★
6 & 7	31.0	8.3	10.2	15.7	★★★★
6 & 8	32.0	11.3	13.8	20.7	★★★★
7 & 8	30.4	10.9	12.9	18.2	★★★★
7 & 9	32.1	8.3	10.2	15.7	★★★★
7 & 10	29.3	8.3	10.2	15.7	★★★★
8 & 9	32.4	11.3	18.8	20.7	★★★★
8 & 10	33.9	11.2	13.5	20.7	★★★★

KEY

★ There is not 90% confidence that the two groups overlap by less than 1%
ie. the two groups overlap

★★ There is 90% but not 95% confidence that the two groups overlap by less than 1%

★★★ There is 95% but not 99% confidence that the two groups overlap by less than 1%

★★★★ The two groups overlap by less than 1% at 99% confidence
ie. the two groups do not overlap

Figure 5.6 Percentage positive characters for each taxon

CHARACTER	PERCENT POSTITIVE FOR EACH TAXON									
	1 n=32	2 n=10	3 n=3	4 n=17	5 n=14	6 n=1	7 n=4	8 n=10	9 n=1	10 n=1
I- Erythritol	29	22	1	6	1	1	75	1	1	1
D- Melibiose	10	1	1	7	1	1	99	1	99	1
Acetic Acid	84	56	33	79	85	1	99	63	1	99
P- Hydroxyphenyl Acetic Acid	87	99	67	1	43	99	75	30	1	1
Bromo Succinic Acid	99	99	67	99	99	99	75	80	99	99
L- Histidine	81	99	33	99	93	1	75	43	1	99
Urocanic Acid	94	90	99	82	79	1	75	44	1	1
A- Cyclodextrin	1	1	1	1	1	1	99	1	1	99
D- Fructose	90	78	67	88	93	1	99	1	1	1
B- Methyl-D-Glucoside	3	1	1	6	1	1	99	1	1	1
Cis- Aconitic Acid	99	99	99	94	99	99	75	40	1	1
Itaconic Acid	53	60	1	12	14	99	1	10	1	1
Succinamic Acid	85	99	67	76	93	99	99	70	1	1
Hydroxy-L-Proline	97	99	99	71	93	99	75	11	1	1
Inosine	99	99	99	60	99	99	99	50	99	99
Dextrin	1	1	1	12	23	1	99	1	99	99
L- Fucose	3	1	1	88	1	1	25	1	1	1
D- Psicose	63	33	1	88	71	1	99	10	1	1
Citric Acid	99	99	99	94	99	99	99	25	1	1
A- Keto-Butyric Acid	40	22	1	33	38	1	75	67	99	99
Glucuronamide	83	1	33	31	15	1	75	1	1	1
L- Leucine	87	99	67	44	99	1	50	50	1	99
Uridine	93	56	1	33	15	1	99	25	99	99
Glycogen	1	1	1	1	1	1	50	1	1	99
D- Galactose	99	70	99	81	21	1	99	1	1	1
D- Raffinose	1	1	1	1	1	1	75	1	1	1
Formic Acid	62	33	1	69	99	1	99	50	1	1
A- Keto-Glutaric Acid	99	99	99	99	99	99	75	44	1	99
Alaninamide	45	70	1	25	86	99	99	14	1	99
L- Ornithine	71	99	67	67	99	99	75	20	1	99
Thymidine	1	1	1	1	1	1	75	20	1	99
Tween 40	94	99	99	88	99	99	99	99	99	99
Gentiobiose	1	1	1	1	1	1	50	1	99	1
L- Rhamnose	13	50	1	1	1	99	50	1	1	1
D- Galactonic Acid Lactone	97	80	99	41	29	99	75	1	1	1
A- Keto-Valeric Acid	19	1	1	31	79	1	50	44	99	99
D- Alanine	99	99	33	76	92	1	99	33	99	99
L- Phenylalanine	6	1	1	6	17	1	75	22	1	99
Phenylethylamine	6	1	33	1	38	1	1	10	1	99
Tween 80	86	99	67	47	99	99	99	70	1	99
A-D- Glucose	99	99	99	99	99	99	99	1	99	99
D- Sorbitol	97	10	33	7	1	1	99	1	1	1
D- Galacturonic Acid	84	1	33	41	14	1	99	1	1	99
D,L- Lactic Acid	99	99	99	99	99	99	99	70	99	99
L- Alanine	99	90	33	99	93	99	99	40	99	99
L- Proline	99	99	99	99	99	1	99	56	99	99
Putrescine	7	10	33	69	99	1	75	1	1	1
N- Acetyl Galactosamine	1	1	1	1	1	1	50	1	99	1

n= number of OTUs

Figure 5.6 Percentage positive characters for each taxon

CHARACTER	PERCENT POSTITIVE FOR EACH TAXON									
	1 n=32	2 n=10	3 n=3	4 n=17	5 n=14	6 n=1	7 n=4	8 n=10	9 n=1	10 n=1
M- Inositol	97	90	67	81	7	1	99	1	1	1
Sucrose	44	1	1	12	15	1	75	1	99	99
D- Gluconic Acid	99	99	99	99	99	99	99	30	1	1
Malonic Acid	81	50	67	1	57	1	75	10	1	1
L- Alanyl-Glycine	99	99	1	88	92	99	99	11	99	99
L- Pyroglutamic Acid	78	99	99	99	93	99	50	30	1	99
2- Amino Ethanol	87	40	33	47	93	99	75	1	1	1
N- Acetyl-D-Glucosamine	58	80	67	6	43	1	99	10	99	1
A-D- Lactose	1	1	1	1	1	1	25	1	99	1
D- Trehalose	59	99	67	13	15	99	99	1	99	1
D- Glucosaminic Acid	99	80	1	1	23	1	99	1	1	1
Propionic Acid	94	80	67	99	86	1	75	78	99	99
L- Asparagine	99	99	99	99	99	99	99	80	99	99
D- Serine	17	50	1	82	36	1	99	11	1	1
2,3- Butanediol	7	1	1	1	1	1	50	1	1	99
Adonitol	67	99	1	1	7	99	50	1	1	1
Lactulose	1	1	1	1	1	1	25	1	99	1
Turanose	15	10	1	27	23	1	99	1	99	1
D- Glucuronic Acid	94	1	33	50	14	1	99	1	1	1
Quinic Acid	99	80	99	40	99	1	50	1	1	1
L- Aspartic Acid	99	99	99	99	99	99	99	56	1	99
L- Serine	99	99	99	76	99	99	99	67	99	99
Glycerol	99	99	99	99	99	99	99	20	1	1
L- Arabinose	99	1	99	99	38	1	99	11	1	1
Maltose	1	10	1	6	1	1	75	10	99	99
Xylitol	7	22	1	1	1	1	99	1	1	1
A- Hydroxybutyric Acid	55	33	1	50	29	1	99	50	99	99
D- Saccharic Acid	99	1	67	69	64	1	99	1	1	99
L- Glutamic Acid	99	99	99	99	99	99	99	78	1	99
L- Threonine	71	44	1	7	77	1	99	38	99	99
D,L-A- Glycerol Phosphate	13	33	1	6	14	99	99	1	1	1
D- Arabitol	97	90	33	1	29	99	75	1	1	1
D- Mannitol	97	80	99	1	57	1	99	1	1	1
Methyl Pyruvate	88	80	33	94	92	1	99	90	99	99
B- Hydroxybutyric Acid	99	99	99	40	99	99	75	43	99	1
Sebacic Acid	1	50	1	1	1	1	1	11	1	1
Glycyl-L-Aspartic Acid	7	10	1	6	23	1	50	1	99	99
Carnitine	85	99	67	35	93	1	75	1	1	1
Glucose-1-Phosphate	1	1	1	1	1	1	50	1	1	99
D- Cellobiose	1	1	1	1	7	1	1	1	99	1
D- Mannose	97	99	67	65	64	99	99	1	99	99
Methyl Succinate	87	99	1	99	93	99	99	67	99	99
G-Hydroxybutyric Acid	3	1	33	1	31	1	50	20	1	99
Succinic Acid	99	99	99	94	99	99	99	78	99	1
Glycyl-L-Glutamic Acid	62	90	33	40	86	99	99	33	1	99
G- Amino Butyric Acid	97	99	99	99	99	1	99	22	1	1
Glucose-6-Phosphate	1	10	1	1	1	1	75	1	1	99

n= number of OTUs

Table 5.4 The top ten most diagnostic character states for each taxon (generated by the DIACHAR program)

	CHARACTER	CHARACTER STATE		CHARACTER	CHARACTER STATE
TAXON 1	D- GLUCOSAMINIC ACID	POSITIVE	TAXON 6	L- PROLINE	NEGATIVE
	D- SORBITOL	POSITIVE		L- RHAMNOSE	POSITIVE
	L- ARABINOSE	POSITIVE		METHYL PYRUVATE	NEGATIVE
	D- GALACTOSE	POSITIVE		PROPIONIC ACID	NEGATIVE
	QUINIC ACID	POSITIVE		D, L-A-GLYCEROL	POSITIVE
	D- GLUCURONIC ACID	POSITIVE		ITACONIC ACID	POSITIVE
	D- MANNITOL	POSITIVE		D- ALANINE	NEGATIVE
	M- INOSITOL	POSITIVE		ADONITOL	POSITIVE
	D- ARABITOL	POSITIVE		G- AMINO	NEGATIVE
	D- SACCHARIC ACID	POSITIVE		L- HISTIDINE	NEGATIVE
TAXON 2	ADONITOL	POSITIVE	TAXON 7	B- METHYL-D-GLUCOSIDE	POSITIVE
	CARNITINE	POSITIVE		XYLITOL	POSITIVE
	D- SACCHARIC ACID	NEGATIVE		A- CYCLODEXTRIN	POSITIVE
	L- ARABINOSE	NEGATIVE		D- MELIBIOSE	POSITIVE
	P- HYDROXYPHENYL ACETIC ACID	POSITIVE		D- SORBITOL	POSITIVE
	D- TREHALOSE	POSITIVE		D, L-A-GLYCEROL PHOSPHATE	POSITIVE
	G- AMINO BUTYRIC ACID	POSITIVE		TURANOSE	POSITIVE
	A- KETO-VALERIC ACID	NEGATIVE		D- GLUCOSAMINIC ACID	POSITIVE
	DEXTRIN	NEGATIVE		D- GLUCURONIC ACID	POSITIVE
	D- GALACTURONIC ACID	NEGATIVE		DEXTRIN	POSITIVE
TAXON 3	METHYL SUCCINATE	NEGATIVE	TAXON 8	A- D-GLUCOSE	NEGATIVE
	L- ALANYL-GLYCINE	NEGATIVE		D- MANNOSE	NEGATIVE
	D- MANNITOL	POSITIVE		D- TREHALOSE	NEGATIVE
	L- ARABINOSE	POSITIVE		D- FRUCTOSE	NEGATIVE
	D- GALACTOSE	POSITIVE		D- GALACTONIC ACID LACTONE	NEGATIVE
	QUINIC ACID	POSITIVE		D- SACCHARIC ACID	NEGATIVE
	ALANINAMIDE	NEGATIVE		D- GALACTOSE	NEGATIVE
	L- THREONINE	NEGATIVE		QUINIC ACID	NEGATIVE
	URIDINE	NEGATIVE		D- MANNITOL	NEGATIVE
	A- HYDROXYBUTYRIC ACID	NEGATIVE		M- INOSITOL	NEGATIVE
TAXON 4	L- ARABINOSE	POSITIVE	TAXON 9	D- CELLOBIOSE	POSITIVE
	P- HYDROXYPHENYL ACETIC ACID	NEGATIVE		L- GLUTAMIC ACID	NEGATIVE
	L- FUCOSE	POSITIVE		A- D-LACTOSE	POSITIVE
	D- MANNITOL	NEGATIVE		LACTULOSE	POSITIVE
	G- AMINO BUTYRIC ACID	POSITIVE		L- ASPARTIC ACID	NEGATIVE
	D- ARABITOL	NEGATIVE		GENTIOBIOSE	POSITIVE
	L- THREONINE	NEGATIVE		N- ACETYL GALACTOSAMINE	POSITIVE
	L- HISTIDINE	POSITIVE		A- KETO-GLUTARIC ACID	NEGATIVE
	ADONITOL	NEGATIVE		D- MELIBIOSE	POSITIVE
	D- GLUCOSAMINIC ACID	NEGATIVE		D- GLUCONIC ACID	NEGATIVE
TAXON 5	PUTRESCINE	POSITIVE	TAXON 10	SUCCINIC ACID	NEGATIVE
	FORMIC ACID	POSITIVE		GLYCOGEN	POSITIVE
	QUINIC ACID	POSITIVE		GLUCOSE-1-PHOSPHATE	POSITIVE
	G- AMINO BUTYRIC ACID	POSITIVE		2, 3- BUTANEDIOL	POSITIVE
	CARNITINE	POSITIVE		A- CYCLODEXTRIN	POSITIVE
	L- LEUCINE	POSITIVE		GLUCOSE-6-PHOSPHATE	POSITIVE
	D- FRUCTOSE	POSITIVE		THYMIDINE	POSITIVE
	2- AMINO ETHANOL	POSITIVE		PHENYLETHYLAMINE	POSITIVE
	GLYCEROL	POSITIVE		L- PHENYLALANINE	POSITIVE
	M- INOSITOL	NEGATIVE		D- GLUCONIC ACID	NEGATIVE

Figure 5.7A Principle co-ordinate analysis 2 dimensional plot

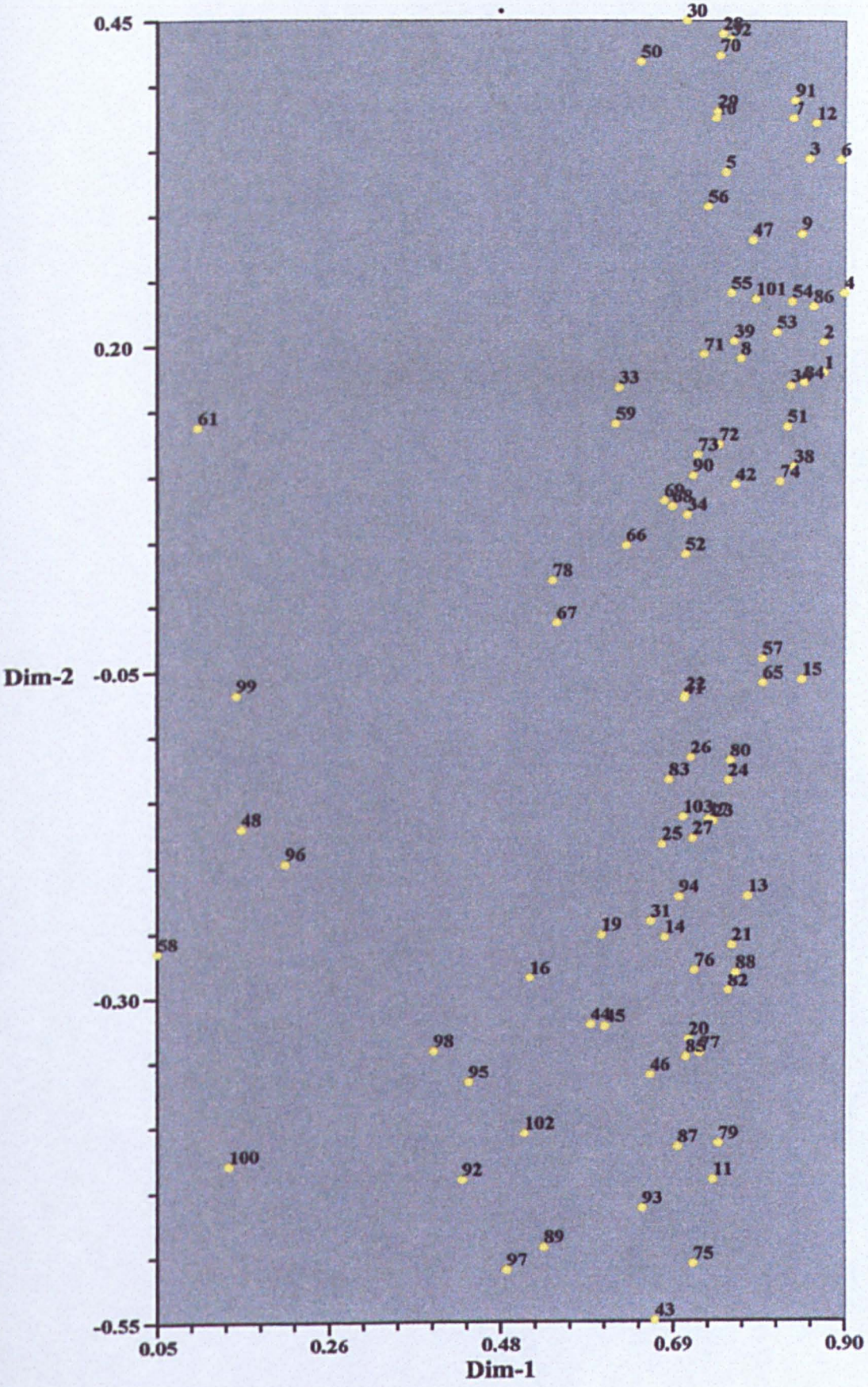


Figure 5.7B Principle co-ordinate 2-dimensional plot

side of the plot are pseudomonads with the exception of *Acinetobacter junii* and *Alcaligenes faecalis*. The OTUs 48 and 96 are not related to the rest of the members of taxon 8 and exist on the non-pseudomonad side of the plot.

A representation of the Principle co-ordinate analysis in three dimensions (X-axis = dimension 1, Y-axis = dimension 2 and Z-axis = dimension 3) is shown in Figures 5.8A and 5.8B. In Figure 5.8B, in which the members of each taxon are colour coded, taxon 1 appears to be very tightly grouped and appears not to overlap with any other taxon. Taxa 2, 3, 4 and 5 are less tightly grouped than taxon 1 and could possibly be divided into sub-groups. Taxa 3, 4 and 5 do not overlap, as they appeared to do in the 2D representation. Taxa 7 and 8 are highly disparate groups where all the members of each group do not belong to the same genus. The Principal co-ordinate analysis clearly reflects the same broad structure as the dendrogram (Figure 5.4) because both are expressions of distance relations.

Figure 5.9 represents the 3-D plot of the principle co-ordinate analyses for the reference strains used during this numerical study. The relatedness between the various *Pseudomonas* species can be seen. The *Ps. fluorescens, Ps.aeruginosa, Ps. putida* and *Ps. reptilovora* species (fluorescent Pseudomonads) are located on the right-hand side of the plot. *Ps. alcaligenes* is located at the front of the plot, behind which is located *Ps. acidovorans* and *Ps. testosteroni*. These three Pseudomonas species are related in that they are known to be less active and free-living. The *Ps. putida* strains 102 and 89 falls outside the fluorescent pseudomonad group and is positioned nearer to the less active pseudomonads.

Figure 5.8A Principle co-ordinate analysis 3-dimensional plot

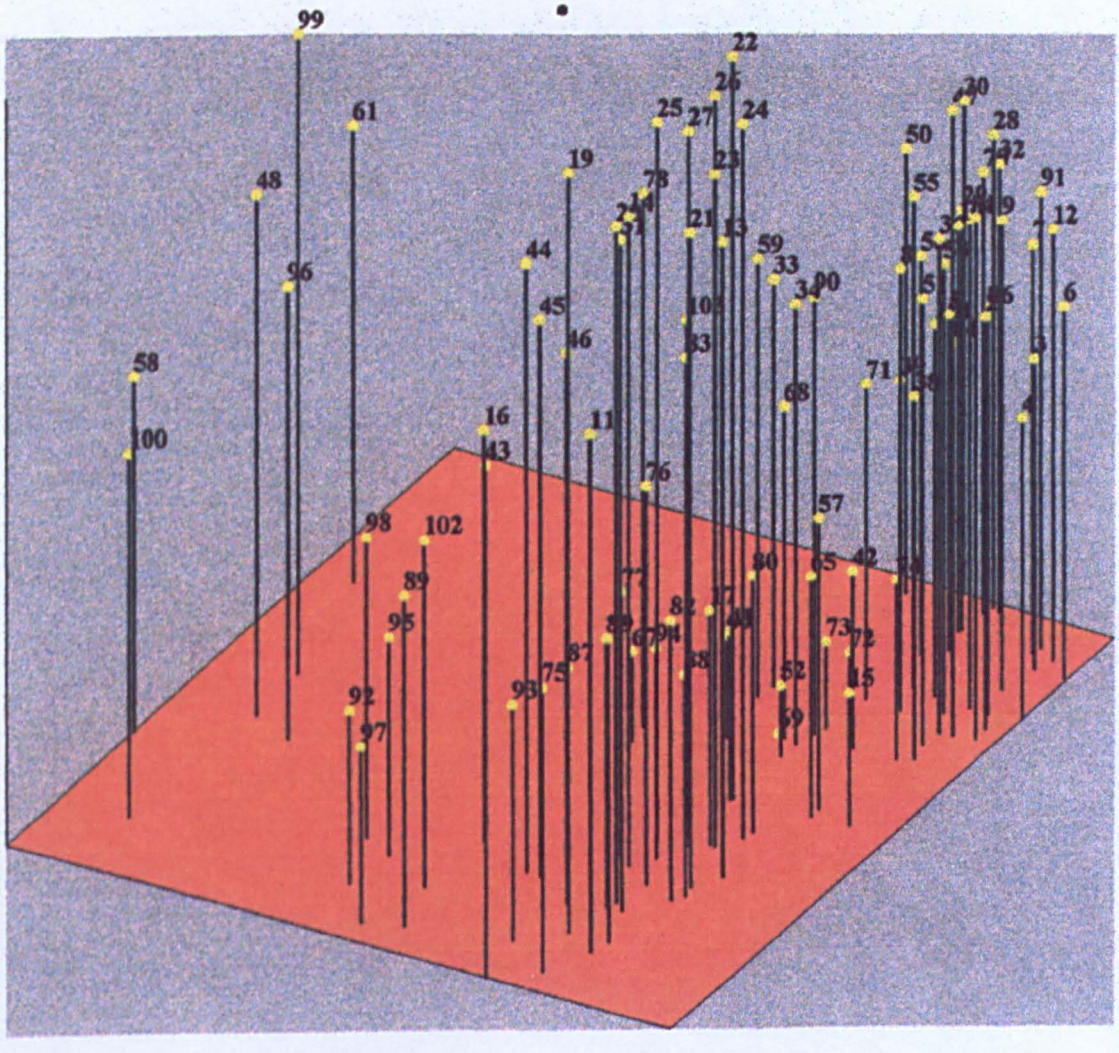


Figure 5.8B Principle co-ordinate analysis 3-dimensional plot

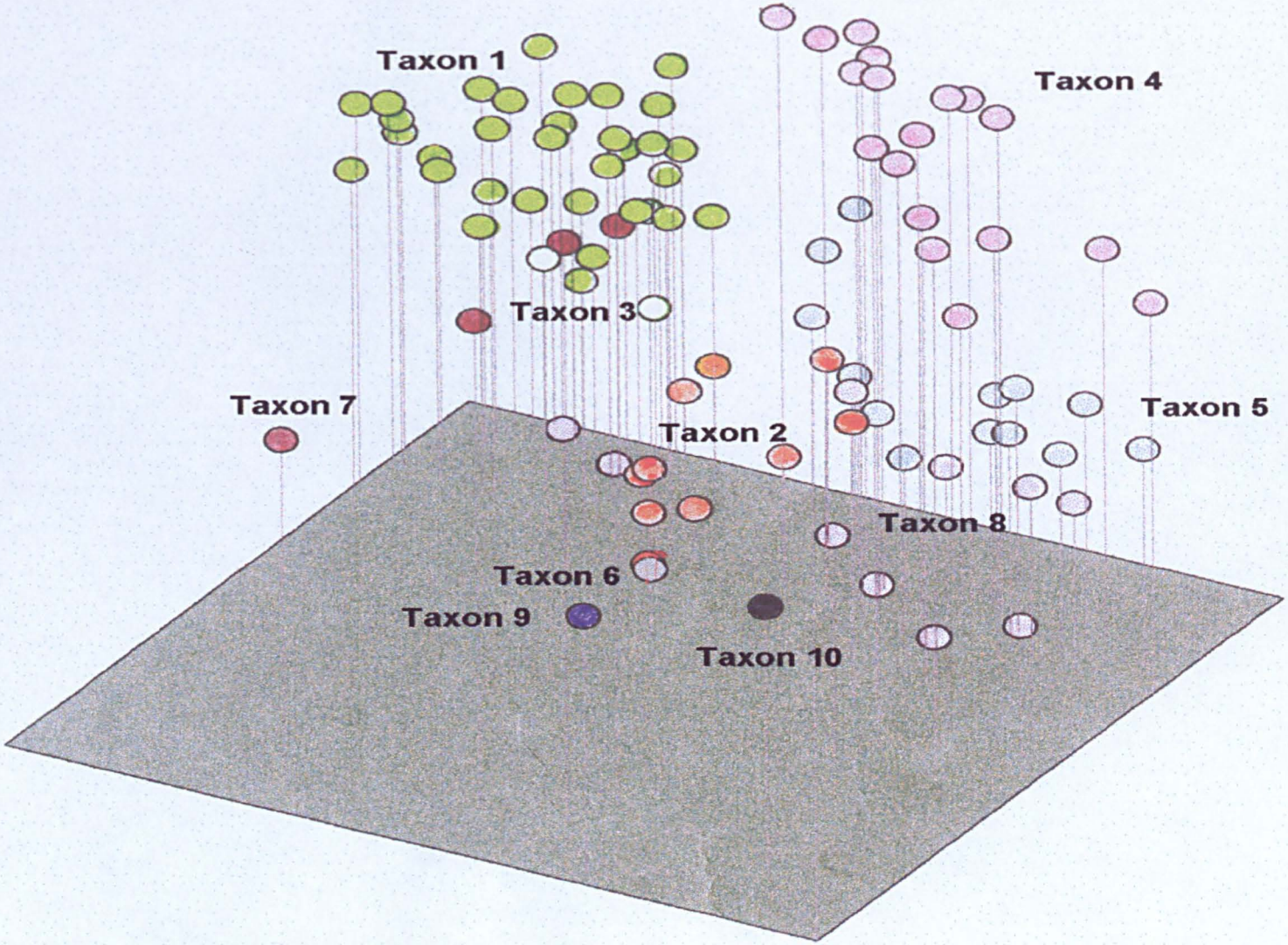
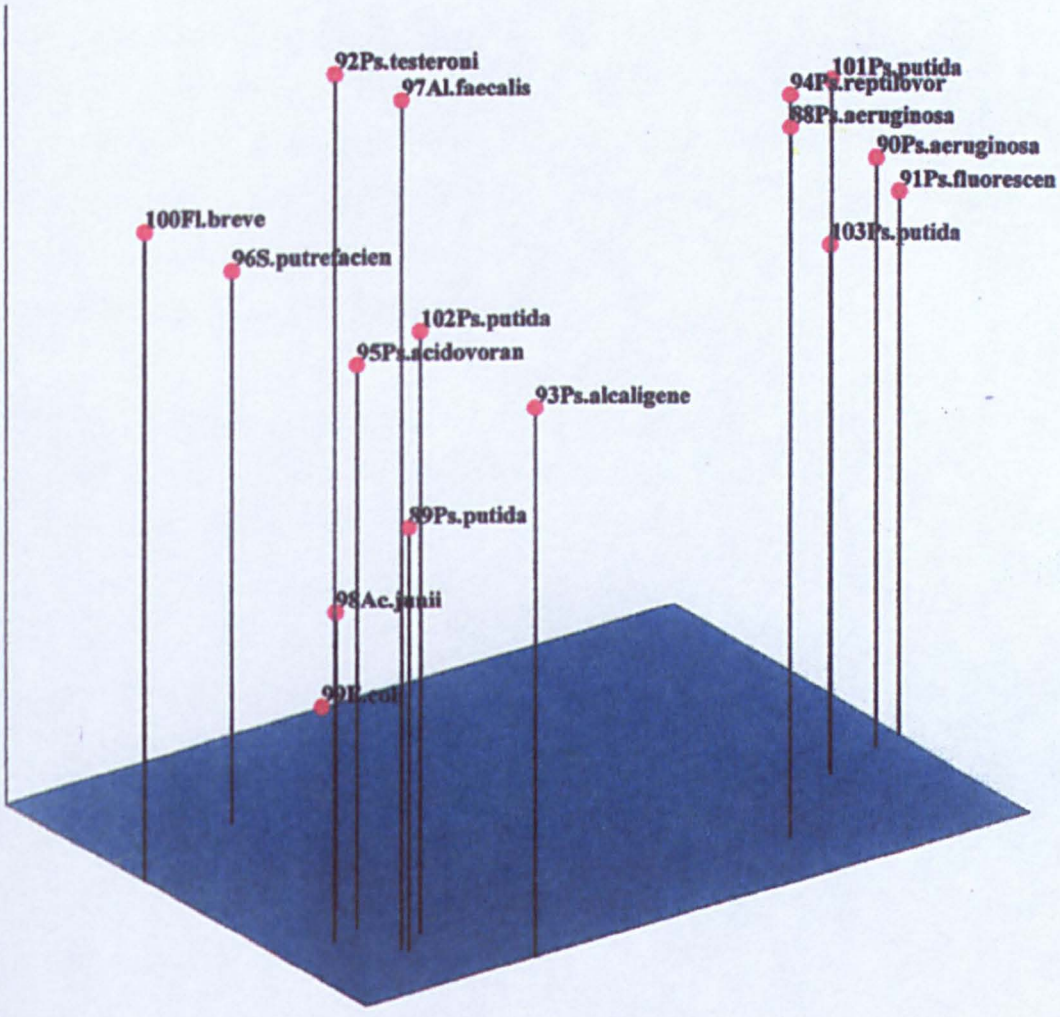


Figure 5.9 3-D plot of principle co-ordinate analysis of the reference strains



5.6 DISCUSSION

Numerical analysis of the phenotypic characters resulted in a number of well-defined clusters. Taxon 1 is the *Ps. fluorescens* group, as it contains the *Ps. fluorescens* NCTC 10038 reference strain. The ordered shaded distance matrix (Figure 6.5) visually represents the group's high degree of relatedness as does the Principle co-ordinate analysis (Figure 6.8B). The API 20NE system named the majority of the organisms in this group as *Ps. fluorescens* but some were labeled as *Pseudomonas* species, *Ps. putida* and *Ps. chloroaphis*. *Ps. chloroaphis* is biovar D of *Ps. fluorescens*. The inclusion of *Ps. putida* strains into the *Ps. fluorescens* group has been shown by Grimont *et al.* (1996), who were working with known strains. The reason for this could be that tests present on the Biolog microplate™ cannot fully discriminate between *Ps. fluorescens* and *Ps. putida*. Alternatively some strains labeled *Ps. putida* may be intermediates between *Ps. fluorescens* and *Ps. putida*.

Taxon 2 contained no reference strains but the group members are mainly *Ps.aeruginosa* or *Ps.fluorescens*. The ordered shaded similarity matrix shows a dark triangle on the hypotenuse of the matrix indicating that the group members are closely related to each other. The 2 and 3D-ordination plot evidence a fairly cohesive group. The API 20NE presumptively identified one taxon member as being *Ps. pseudomallei*. On further examination this may prove to be a misnomer. *Ps. pseudomallei* species is classified as a RNA group II pseudomonad whereas the fluorescent pseudomonads belong to RNA group I. The RNA group II and I pseudomonads are too distantly related to each other to be incorporated into the same taxon. Taxon 3 is a *Ps.aeruginosa* group whose cohesiveness is clearly shown in the 3D-ordination plots. The *Ps. fluorescens* present in this group may be a biovar that is closely related to *Ps. aeruginosa*.

Taxon 4 did not contain a reference strain but *Ps. putida* was the predominate organism in this group. The ordered distance matrix showed that the group could be further divided into two sub-groups one of which was made up entirely of bacteria from raw milk. The OVERMAT statistical analysis showed that the taxa did not overlap with taxon 5 or 8 but overlapped slightly with taxon 7 (less than 1% at the 95% confidence limit).

One of the sub-groups of taxon 5 contained the *Ps. aeruginosa* NCTC 10332 type strain and the other the *Ps. reptilovora* IFR 461 marker strain. Isolates 17, 75, 87 and 77 can be considered to be *Ps. aeruginosa* strains and the other members of this taxon seem to be

biovars of *Ps. fluorescens* with the exception of *Ps.putida* and a presumptive *Ps. cepacia*. *Ps. cepacia* is a nutritionally versatile RNA group II pseudomonad with a noteworthy mechanism for foreign gene recruitment.

The four members of taxon 7 naturally split into two sub-groups one being a *Ps. fluorescens* group and the other contained non-pseudomonads. The ordination plots indicate that this taxon is not a cohesive group. The ordered distance matrices indicate that the two *Ps. fluorescens* species are closely related to each other and form a sub-group that ties at the 7% distance level. The OVERMAT overlap program failed to generate statistically significant data regarding the overlap between taxon 1 and 7, possibly due to the disparity between group membership numbers. The statistical analysis does indicate that this taxon overlaps by less than 1% at the 95% confidence limit with the *Ps. putida* taxon (taxon 4) and also with taxon 5. The members of this taxon illustrate that bacteria of different genera, which originate from the same source, can give similar nutritional signatures.

Taxon 8 is a non-cohesive group as shown by the shaded distance matrix and the 2 and 3D-ordination plots. The members of taxon 8 not only contain known strains of different genera but RNA group I (*Ps. putida* and *Ps. alcaligenes*), group II (*Ps. picketti*) and group III (*Ps. acidovorans* and *Ps. testosteroni*) pseudomonads too.

Pseudomonas putida was defined by Stanier *et al.* (1966) as forming two biovars A and B. Palleroni *et al.* (1972) demonstrated that the two biovars were not closely related, as biovar B has a close affinity with *Ps. fluorescens*. Biovar A was considered by Palleroni *et al.* (1972) to be typical of *Ps. putida* but with respect to DNA homology was found to be very heterogeneous. The same conclusion regarding the demonstration of heterogeneity within the *Ps. putida* species was found in this taxonomic analysis. Within the highly cohesive *Ps. fluorescens* group (taxon 1) three presumptive *Ps. putida* isolates were grouped. These *Ps. putida* isolates could be biovar B. Those *Ps. putida* isolates found in the *Ps.putida* group (taxon 4) could be biovar A. Those examples of *Ps. putida* that are distributed in taxon 5 (isolate code 103) and taxon 8 (isolate code 89 and 102) would therefore also belong to biovar A. The wide distribution of *Ps. putida* strains throughout the taxa in this analysis, defined by nutrient utilisation, reflects the known heterogeneity that exists within *Ps. putida* species. To establish definitively the description of

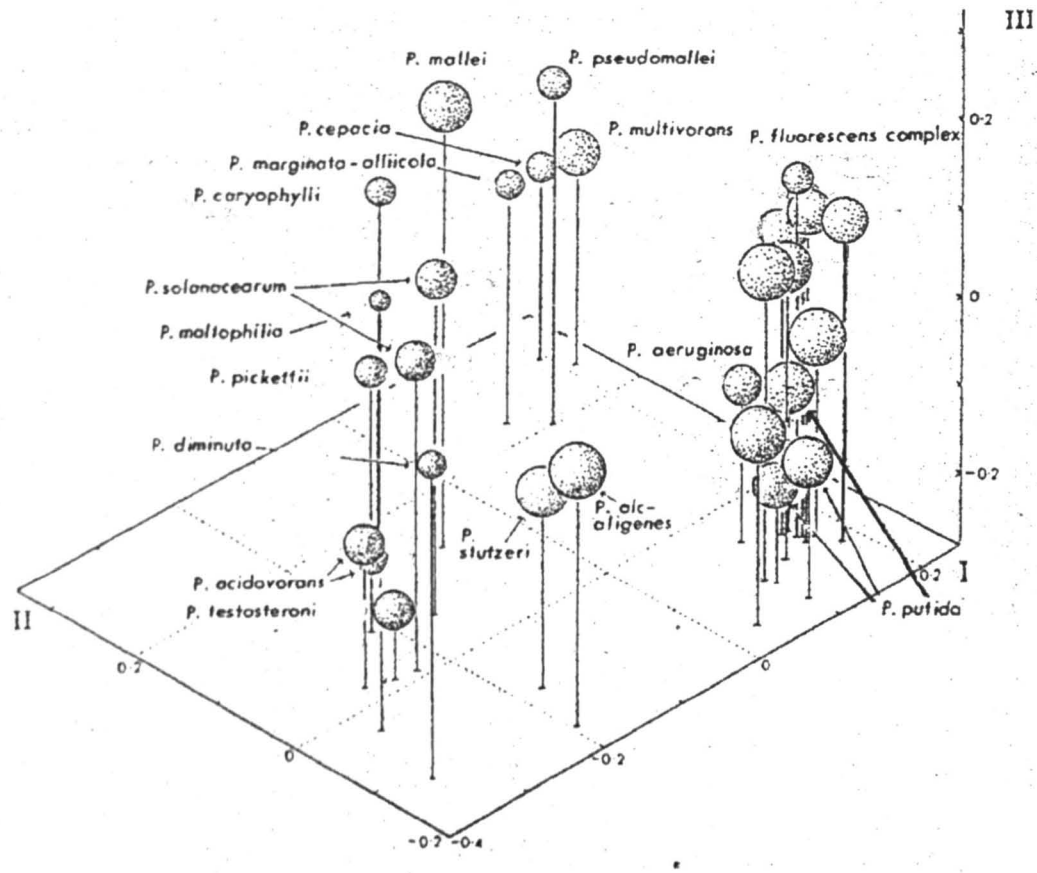


Figure 5.10 Principle co-ordinate analysis of the Euclidean distances between 29 *Pseudomonas* groups as derived by Sneath *et al.* (1981)

each *Ps. putida* biovar further work would be necessary, which would incorporate biovar A and biovar B reference strains.

A similar and distinctly more complex situation exists for *Pseudomonas fluorescens* which species has 5 biovars (I- V)(Palleroni *et al.* 1972). In this study *Ps. fluorescens* isolates were present in six out of ten taxa. Grimont *et al.* (1996) found that strains labeled *Ps. fluorescens* were found in six phenons out of twenty-nine, using the Biolog system. The results are consistent with Sneath *et al.* (1981).

The percentage positive characters for each group (Figure 6.6) defines each taxon by its member's nutritional requirements. A phenotypic "fingerprint" is generated for each taxon that highlights characters that distinguish between groups. When looking at the relationship between phenotypic diversity and strain source the percentage positive characters would be very useful. Theoretically, presumptive identifications of unknown isolates could be made using the diagnostic character state information and would be a rapid way of identifying specific species and biovars.

In 1981 Sneath *et al.* initiated Principal Co-ordinate Analysis based on published records of substrate utilisation for *Pseudomonas* (using 155 characters and 401 OTUs). Twenty-nine groups (relating to species) were distinguished (Figure 6.9). The relative positions of the *Ps. fluorescens*, *Ps. aeruginosa* and *Ps. putida* clusters in the three dimensional plot were in accordance with the relative positioning attained in this investigation for taxon 1, taxon 5 and taxon 4. The relative positions of the reference strains *Ps. alcaligenes* NCTC 10367, *Ps. testosteroni* NCTC 10698 and *Ps. acidovorans* NCTC 10683 were also in accordance with the results of Sneath *et al.* (Figure 6.10). The numerical analysis revealed that members of sub-groups that formed within taxa usually were isolated from the same source which is an occurrence that has been noted by other workers (Sneath *et al.* 1981 (with *Pseudomonas*) and Thompson, 1996 (with *Bacillus*)).

The majority of the environmental isolates studied were rRNA group I. As of April 1996 rRNA group I pseudomonads are "true" *Pseudomonas* species (of which there are about 30 species). The other RNA groups (II-V) have been placed into other genera (e.g. *Ralstonia*, *Burkholderia*, *Comamonas* and *Acidovorax*).

5.7 CONCLUSION

The computer-assisted numerical technique, with the underlying Adansonian principle that every feature should have equal weight, was adopted with the goal of deriving a systematic arrangement of relatedness for *Pseudomonas*-like organisms isolated from spoiled food and the environment. This goal was attained with the use of the D^2/UPGMA algorithm and the binary data generated from the Biolog GN microplate™.

The use of conventional carbon source utilisation tests has in the past been limited by the tediousness of media preparation and replica plating. For diagnostic purposes however, a phenotypic description of bacterial taxa is still very useful. The format of commercial systems, such as the Biolog GN microplate™, makes nutritional studies of scale simpler, faster and more standardised.

The numerical taxonomy study allowed the isolates not identified (and those strains that are possibly misnomers) by the API 20 NE system to be given identification based on their clustering with reference strains. A clearer picture could be drawn from the data with the inclusion of additional *Pseudomonas* reference strains. The results attained efficiently uncovered some of the taxonomic diversity of the pseudomonads and compared well with results produced by other workers in this field.

USE OF ANTIBODIES TO FOLLOW SPOILAGE OF MILK AND DAIRY PRODUCTS

6.1 AIM

The aim of this study was to use the antibody-probes developed to follow the changes in naturally occurring and introduced *Pseudomonas* populations throughout psychrotrophic spoilage of milk products. Dairy products were used as the heterogeneous food system in which to monitor *Pseudomonas* growth. Changes in target population numbers that were detected using the antibody-probes were compared to population numbers obtained from solid agar plate counts.

6.2 INTRODUCTION

Storage of milk products at refrigeration temperatures favours the growth of psychrotrophic bacteria. At low temperatures, growth is usually slow with a long lag phase. The Gram negative *Pseudomonas* spp. usually represents 10% of the microflora of freshly drawn milk, but at the end of product shelf-life, they are the most numerous members of the psychrotrophic population. Whilst growing in raw and processed milk pseudomonads produce an array of proteolytic and lipolytic enzymes that are heat stable and can survive pasteurisation. Hence, the pseudomonads can greatly influence the keeping qualities and spoilage properties of commercial milk and milk products.

The result of failure to control microbial spoilage by food processors is economically very costly. To avoid expensive mistakes, it is important that food manufacturers can accurately predict the behaviour of micro-organisms in their products. Introduced organisms in liquid axenic cultures currently form the basis for predictive models. Following the growth of natural flora within heterogeneous food systems may lead to a better understanding of microbial growth within a complex environment. Investigating the growth of introduced organisms within real food systems and the growth of natural flora may yield valuable data regarding the validity of using introduced organisms (spiking) as a basis for challenge testing.

In this study a range of milk products were spoiled at 4°C. The products included pasteurised cream, pasteurised full-fat, pasteurised semi-skimmed and raw milk. The dairy products that were evaluated were natural oil-in-water emulsions with varying amounts of fat, ranging from 1.7% in semi-skimmed milk to 19.8% in cream. As discussed previously high fat content within an oil-in-water emulsion may affect the behaviour of growth of bacteria within foods (Brocklehurst *et al.*, 1995).

Antibodies raised against *Ps.fluorescens*, *Ps. putida* and *Ps.aeruginosa* isolated from psychrotrophically spoiled food (Chapter 3) were used to monitor the changes in a *Pseudomonas* sub-population throughout the natural psychrotrophic spoilage of milk products. The number of organisms cross-reacting with an antiserum (assessed using colony lifts; Fig.6.1) were compared to counts obtained from *Pseudomonas* CFC agar and nutrient agar. Throughout spoilage, various parameters were monitored, including pH and changes in smell or appearance. The proteolytic activity of *Pseudomonas* colonies was appraised.

In addition milk samples were inoculated with an introduced organism, *Ps. aeruginosa*, and the changes in the target population were monitored with anti-*Ps. aeruginosa* antibodies throughout psychrotrophic spoilage. *Ps. aeruginosa* is not commonly found in cold stored milk (Ternstrom *et al.*, 1993).

The microbial flora obtained from raw and pasteurised milk products spoiled at 4°C and 20°C were evaluated. Colonies exhibiting different morphologies on non-selective agar plate were phenotypically and biochemically characterised.

6.3 METHODS

6.3.1 MILK PRODUCTS

Samples of raw milk were collected, mid flow, in sterile bottles from the bulk tank at the Kingston-upon-Soar farm, Leicestershire. The milk samples were transferred to the laboratory on ice in a cool box. Cartons of pasteurised semi-skimmed milk (1.7% fat), full-fat milk (4.1% fat) and single cream (19.8% fat) were purchased from a local supermarket. Milk product samples that were within five days of the best before date were not chosen.

6.3.2 FOLLOWING THE NATURAL *PSEUDOMONAS* SPOILAGE POPULATION WITH ANTIBODIES

Milk products were allowed to spoil psychrotrophically for up to 15 days at 4°C. Aliquots (1 ml) of each sample were aseptically removed at daily intervals throughout the spoilage period and serially diluted in maximum recovery diluent (MRD; section 2.1). Each dilution was spread in duplicate onto *Pseudomonas* CFC agar (*Pseudomonas* count) and nutrient agar (total viable count). Colony lifts were taken from duplicate nutrient agar plates (with 30-300 colonies) using Hybond-C nitro-cellulose filters (Amersham International, Buckingham, UK; Figure 6.1). Colony lifts were conducted at selected time intervals during spoilage for each product. The filters were then immuno-blotted in the presence of anti-*Pseudomonas* antisera (section 2.11). The Xavier antiserum raised against a *Ps. fluorescens* raw milk isolate was used and non-immune serum was used as a control.

6.3.3 MEASUREMENT OF pH

The pH of the milk products was monitored at intervals throughout spoilage using pH indicator strips (BDH Ltd., Dorset, UK), as per manufacturer's instructions. Two strips were dipped into each aliquot of the aseptically removed test sample.

6.3.4 SENSORY PROPERTIES OF MILK PRODUCTS

An organoleptic assessment was made of an aliquot of test sample at intervals throughout spoilage. Changes in colour and smell were documented.

6.3.5 COMPOSITION OF THE MICROBIAL FLORA IN MILK PRODUCTS

Throughout spoilage (9-15 days at 4°C) aliquots (1 ml) of each milk product were serially diluted in MRD and spread (0.1 ml) in duplicate onto nutrient agar plates. The agar plates were incubated (25°C for 48 h) and colonies that exhibited differing morphologies were sub-cultured onto nutrient agar plates and further characterised. The colonies were assayed for Gram reaction, spore production, motility, oxidase production, fermentation and type of respiration (Chapter 2).

6.3.6 PROTEOLYTIC ACTIVITY OF *PSEUDOMONAS* ISOLATES

Single colonies isolated on *Pseudomonas* CFC agar during spoilage of each milk product were aseptically removed with a sterile toothpick and stabbed into a skim milk agar plate (section 2.1). Care was taken not to stab all the way through the agar. The skim milk

agar plates were then incubated at 4°C and 30°C. Proteolysis was deemed to have occurred if, on growth of the cells, a zone of clearing of the agar medium was seen around the stab point.

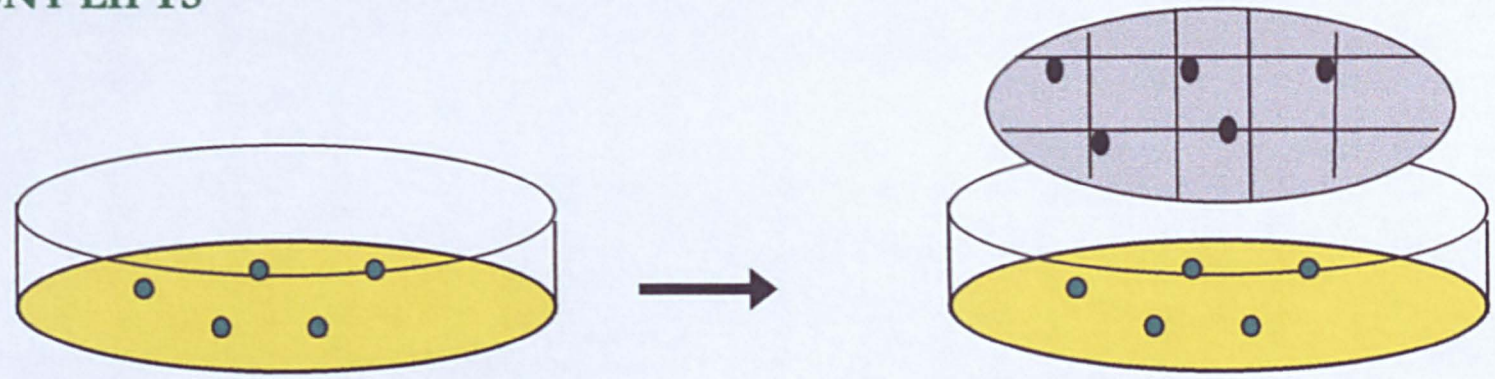
6.3.7 CROSS-REACTIVITY OF *PSEUDOMONAS* ISOLATES WITH ANTISERA

Single colonies of presumptive *Pseudomonas* isolates (as section 6.3.6) were aseptically transferred to a nitro-cellulose membrane and immuno-blotted (section 2.11) with each of Egbert and Xerxes (both raised against *Ps. putida* isolates) and Edward and Xavier (both raised against *Ps. fluorescens* isolates). A negative control (*Acinetobacter junii*) and a positive control for each antiserum was added to each filter. The test isolates were also screened with non-immune serum (NIS). Each serum was used at their respective working dilutions (Chapter 3).

6.3.8 FOLLOWING AN INTRODUCED *PSEUDOMONAS* POPULATION

Reconstituted skimmed milk (reconstituted as per manufacturer's instructions [Oxoid, Basingstoke, UK] and filter sterilised with 0.45µm filter [Gelman Sciences, Michigan, USA]) and pasteurised semi-skimmed milk were inoculated with 5 x 10^3 *Ps. aeruginosa* NO31 cells per ml of milk. The NO31 isolate was previously isolated from spoiled raw chicken and was not previously found to be part of the microflora of the milk products assayed. Uninoculated milk samples were used as controls. Experimental samples (500 ml) and controls were stored at 4°C for up to 8 days. Aliquots (1 ml) of pasteurised milk samples were taken at daily intervals and serially diluted in MRD and spread in duplicate onto *Pseudomonas* CFC and nutrient agar. The sterile skimmed milk and the controls were spread in duplicate onto nutrient agar only. The agar plates were incubated at 25°C for 24 hours. Colony lifts were taken from nutrient agar and *Pseudomonas* CFC spread plates in duplicate that originated from the inoculated and uninoculated semi-skimmed milk samples. The filters were immuno-blotted (section 2.11) using Xtra (anti-*Ps.aeruginosa*) antiserum.

COLONY LIFTS



Agar spread plate

Colonies lifted onto a nitrocellulose filter

- Blocked in PBS + 4% Tween 20
- Incubated with primary antibody
- Washed 3 times in PBST
- Incubated with secondary antibody
- Washed 3 times in PBST
- Colour developed using DAB

IMMUNOBLOTTING

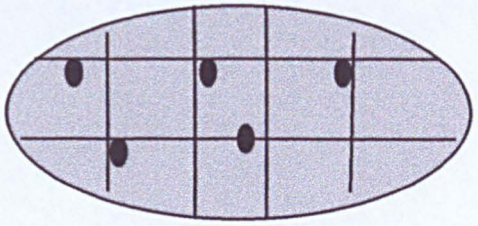


FIGURE 6.1 DIAGRAMMATIC REPRESENTATION OF COLONY LIFTS

6.4 RESULTS

6.4.1 SCREENING THE NATURAL *PSEUDOMONAS* POPULATION WITH XAVIER ANTISERUM

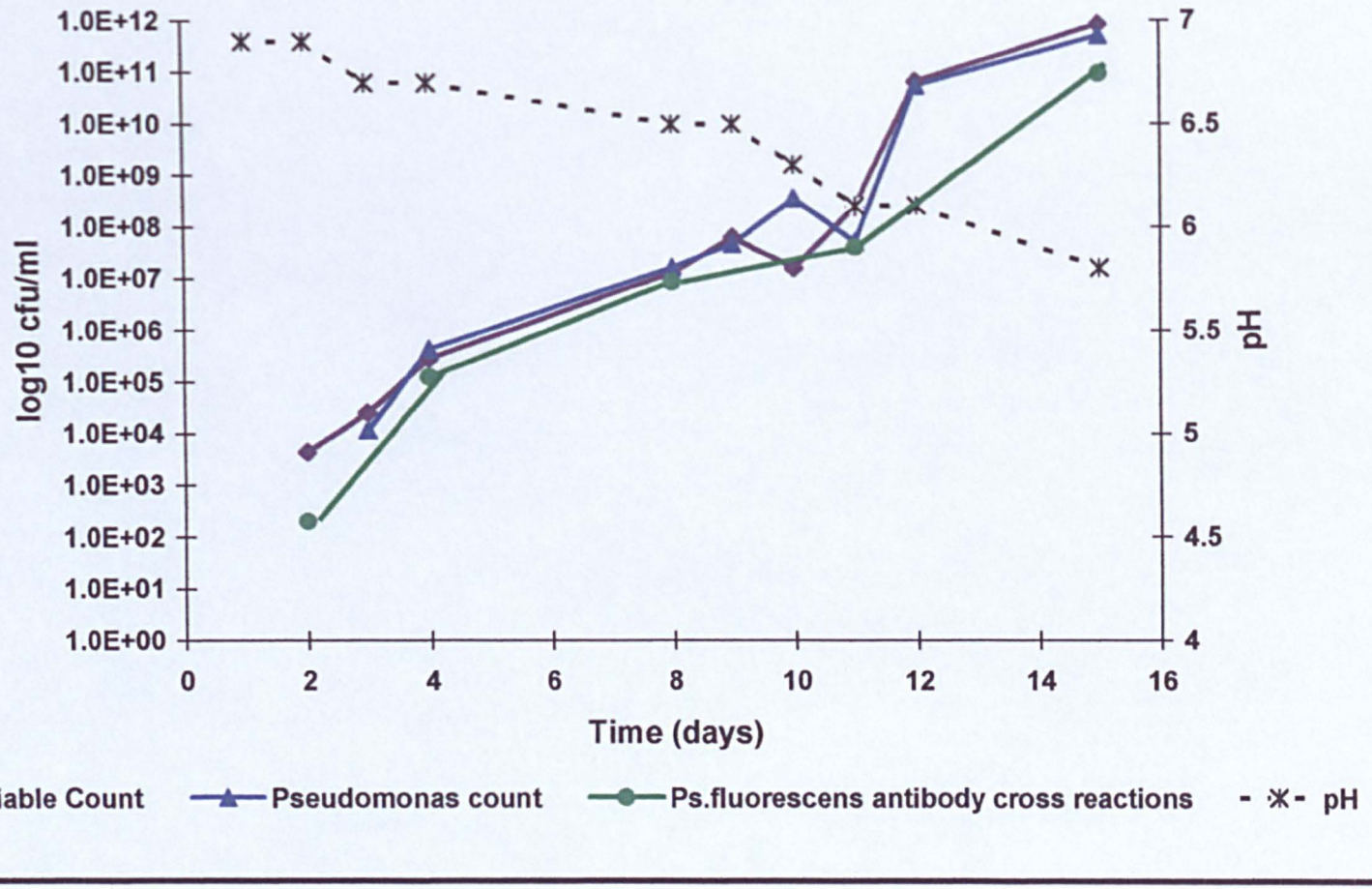
The growth of the psychrotrophic spoilage microflora of pasteurised semi-skimmed milk is shown in Fig. 6.2. The growth curve of the total aerobic population was mimicked closely by the growth curve of the total *Pseudomonas* population. The growth curve of the population of *Pseudomonas* that cross-reacted with the *Ps. fluorescens* antiserum also imitated that of the TVC and the *Pseudomonas* count. Spoilage of semi-skimmed milk was due to *Pseudomonas fluorescens*. Over the spoilage period there was a gradual decrease in pH from 6.9 to 5.8. The microbial flora attained counts of 10^{11} cfu/ml by the end of spoilage.

During the spoilage of full fat milk the pH gradually declined from 6.7 to 6.5 until day 10 (Figure 6.3). From day 10 to day 15 the pH fell quickly to 5.3. The *Pseudomonas* count closely followed that of the total viable count particularly after day 8. The *Pseudomonas* population recognised by the antiserum closely followed that of the TVC and the *Pseudomonas* count in the later stages of spoilage (after day 8). *Pseudomonas* species were dominant in the latter stages of spoilage. The microbial population attained counts of 10^{11} cfu/ml by the end of the spoilage period.

The spoilage trends of pasteurised full fat milk and semi-skimmed milk were similar with a long lag phase that lasted 8 and 10 days respectively. Full fat milk had an exponential phase that was of longer duration than that seen with semi-skimmed milk.

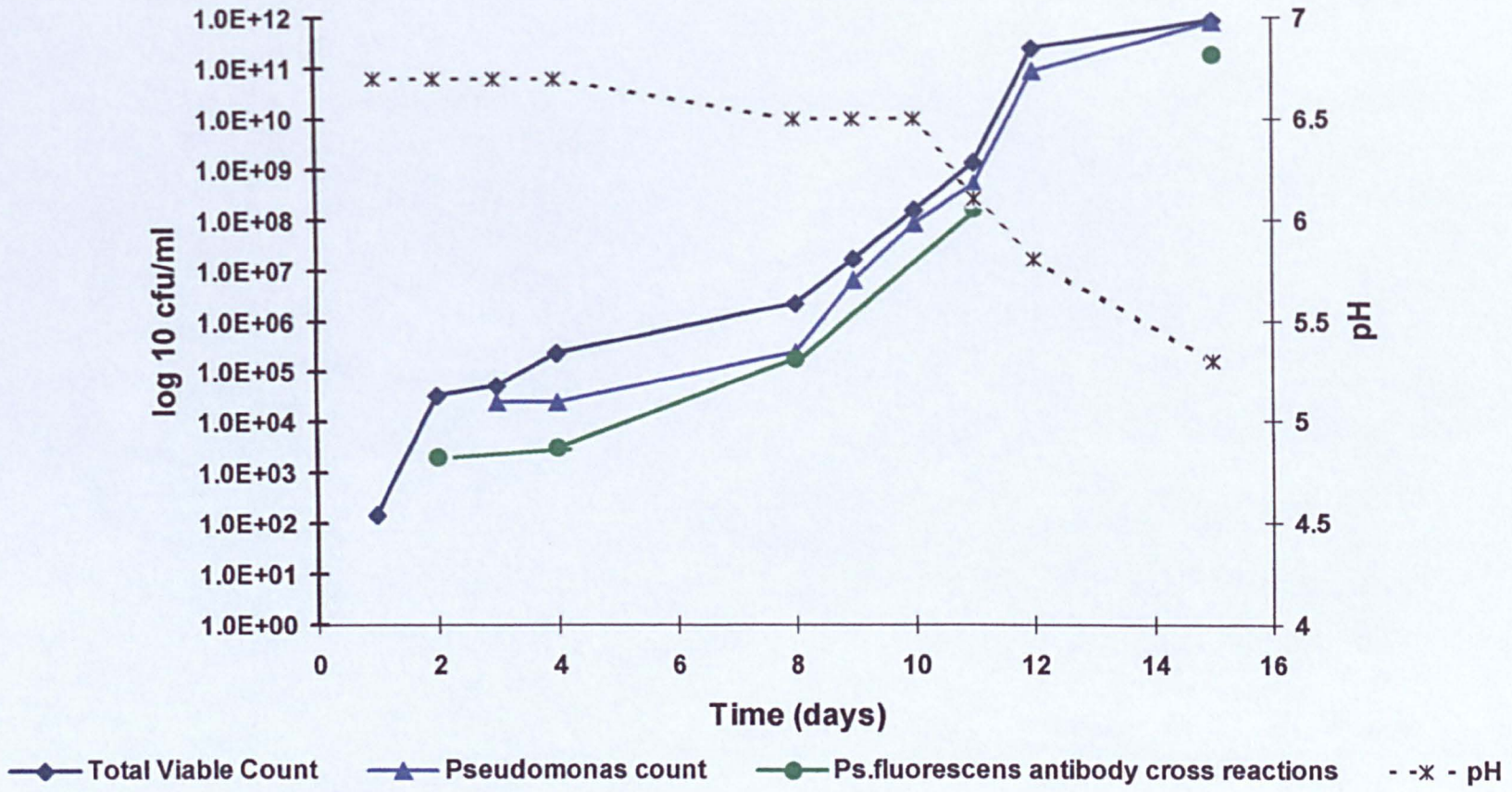
Throughout the first 8 days of spoilage of single cream the pH remained constant at 6.5 and over the next four days fell to 5.8 where it remained until day 15 (Figure 6.4). No significant difference was seen between the TVC, *Pseudomonas* count and the *Pseudomonas* population recognised by the antiserum during the early and mid stages of growth. At the end of spoilage the difference between the *Pseudomonas* count and the population recognised by the antiserum were significantly different.

Figure 6.2 Psychrotrophic Spoilage of Semi-skimmed Milk



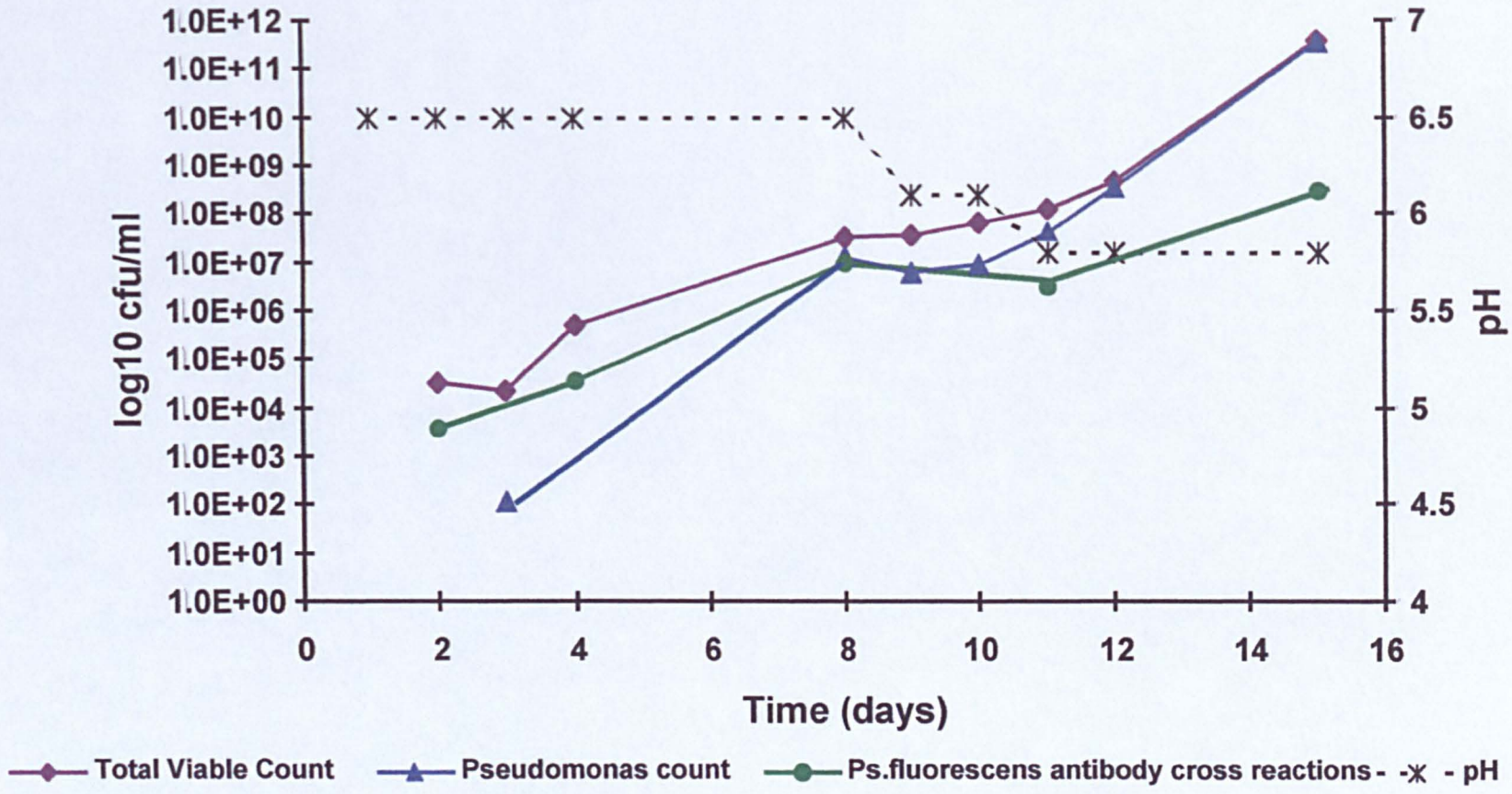
Maximum standard deviation 0.44

Figure 6.3 Psychrotrophic Spoilage of Full Fat Milk



Maximum standard deviation 0.39

Figure 6.4 Psychrotrophic Spoilage of Single Cream



Maximum standard deviation 1 07

The difference in counts suggests the presence of a large population of non-cross-reacting *Pseudomonas* species.

Figure 6.5 show the psychrotrophic spoilage of raw milk. The microbial load of the raw milk at day 1 was 1.15 x 10^5 cfu/ml. The number of bacteria in the raw milk cross-reacting with the antiserum is a small proportion of the total population at the early stages of spoilage. By day 8 the sub-population recognised by antiserum dominates the spoilage microflora and the *Pseudomonas* count indicates the predominance of *Pseudomonas* species within the spoilage microflora. The gradual decrease in pH throughout spoilage was similar to that seen with full fat milk with the exception that a fall in pH was seen earlier at day 4. No lag phase was seen for the TVC and *Pseudomonas* count.

Anomalies were seen during experimentation when the number of organisms cross-reacting with *Pseudomonas* antiserum appeared to bind to more colonies than appeared on the *Pseudomonas* count plate (Fig.6.5). The anomalies were due either to small colonies, that were not clearly visible, cross-reacting with antiserum or more likely consistent experimental counting error.

6.4.2 SENSORY OBSERVATIONS

The changes seen in the appearance and smell of each milk product is summarised in Table 6.1. The onset of a sour smell or thickening of the product indicated that the product had "gone off" and was no longer fit for consumption. Semi-skimmed milk and full fat milk were no longer fit for consumption after 8 days. The single cream and raw milk products could not be consumed after 3 days.

6.4.3 CHARACTERISATION OF SPOILAGE MICROFLORA

Colonies of different morphologies isolated from milk products throughout spoilage were categorised into groups on the basis of the results of their phenotypic and simple biochemical characteristics. The percentage of isolates from spoiling milk products (at 4°C and 20°C) falling into each category is shown in Table 6.2.

In general, at 4°C a wider variety of genera within the microflora of the milk products were seen than at 20°C. Colony type 1 (*Pseudomonas*), colony type 2 (*Bacillus*), colony type 4 (Enterobacteriaceae) and colony type 8 (unknown) were found in all products at 4°C. Colony type 5 (*Acinetobacter*) and 6 (*Micrococcus*), if found, occurred as a very small proportion of the total. Colony type 1 was detected at high proportions of the total isolates characterised but colony type 4 was found in higher proportions at 4°C. The identification of the organism within each category was not confirmed and was given as examples of organisms that fitted the category description.

Type 4 organisms were found, as a large percentage of the isolates chosen, in all products at both spoilage temperatures. At 20°C type 4 organisms were the major type of colony screened in the milk products. Type 4 organisms were predominantly isolated in full fat milk at 20°C. Some members of colony group 4 were spread onto *Pseudomonas* selective agar and was found to be able to grow on it. Colony type 5 & 6 were only found in semi-skimmed milk at 20°C.

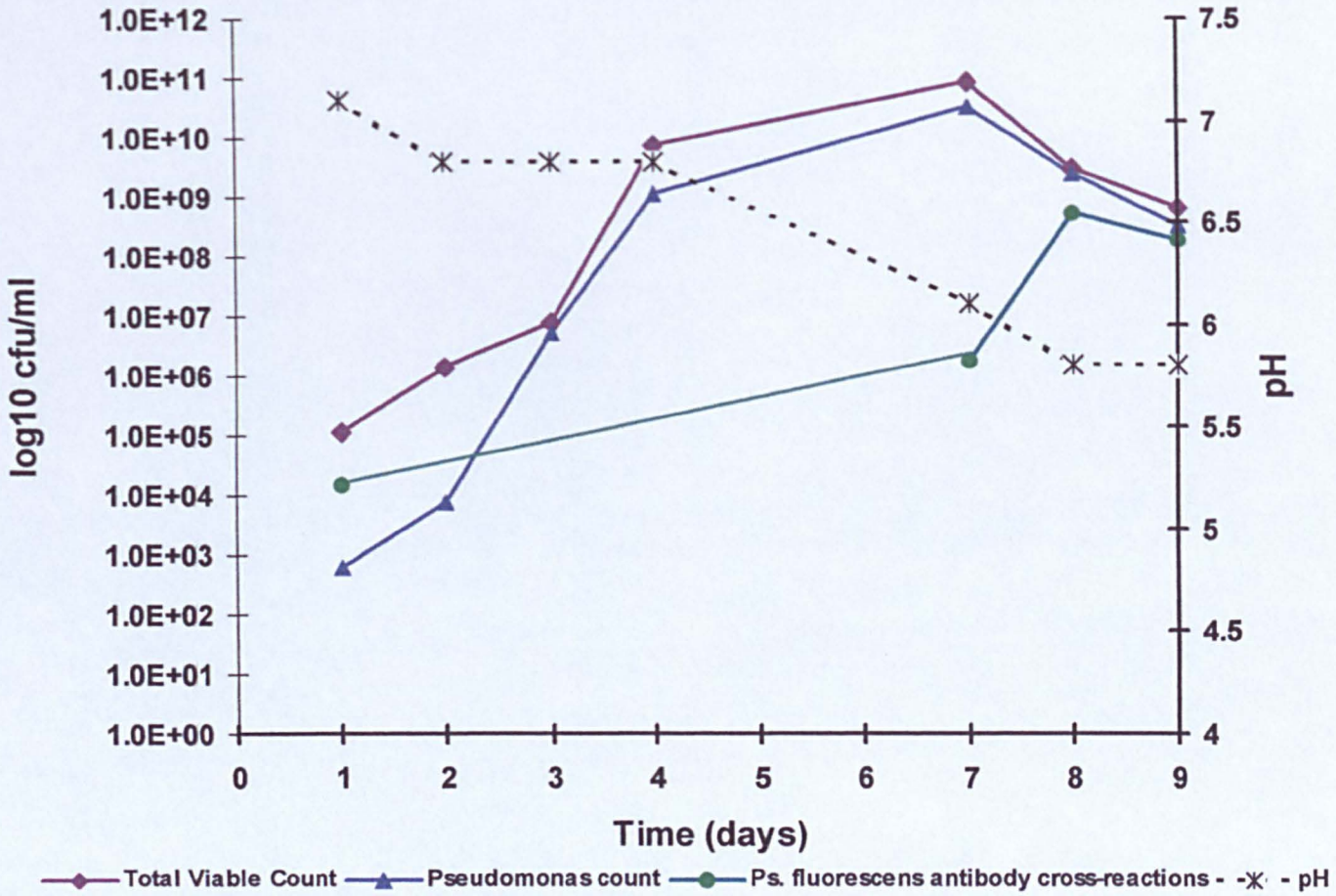
6.4.4 PROTEOLYTIC ACTIVITY OF COLONY TYPE 1 ORGANISMS

A total of 72 colony type 1 organisms (*Pseudomonas*) were isolated from the milk products and assessed for proteolytic activity on skim milk agar and the results are summarised in Table 6.3. The zones of clearing around each test point on the skim milk agar ranged from transparent, where all the available protein was hydrolysed, to semi-opaque, where a proportion of the total protein complement was hydrolysed. Of the 16 isolates tested from raw milk 10 were proteolytic at 4°C and 2 were proteolytic at 30°C. Full fat milk isolates produced similar results to those seen with raw milk isolates, with 7 proteolytic isolates at 4°C and 2 at 30°C. The same organisms did not show proteolytic activity at both temperatures. Little or no proteolytic activity was seen with the isolates from semi-skimmed (at both temperatures) or single cream.

6.4.5 CROSS-REACTIVITY OF COLONY TYPE 1 ORGANISMS WITH *PSEUDOMONAS* ANTISERA

Seventy-two presumptive *Pseudomonas* (cell type 1) isolates from the milk products were immuno-blotted in the presence of Edward, Xavier, Xerxes and Egbert antisera (NIS used as a control) and results are shown in Figure 6.6.

Figure 6.5 Psychrotrophic Spoilage of Raw Milk



Maximum standard deviation 0.43

MILK PRODUCTS	OBSERVATIONS
SEMI-SKIMMED	Day 8 sour smell Day 9 granular texture and became thicker Day 10 separation into two layers, the thin layer had green hue
FULL FAT	Day 8 sour smell and thickened consistency Day 11 separation into thick opaque layer with a thin translucent green layer
SINGLE CREAM	Day 3 thickened Day 4 thickened further with sour smell
RAW MILK	Day 3 thickened Day 4 sour smell Day 7 separation into thick creamy layer and thin translucent layer Day 8 total change in consistency, became thin and watery

TABLE 6.1 SENSORY CHANGES IN MILK PRODUCTS DURING SPOILAGE AT 4°C

COLONY TYPE	PERCENTAGE OF CHARACTERISED ISOLATES OF EACH COLONY TYPE ISOLATED AT EACH TEMPERATURE						
	SEMI-SKIMMED MILK		FULL FAT MILK		SINGLE CREAM		RAW MILK
	4°C n=50	20°C n=50	4°C n=50	20°C n=50	4°C n=50	20°C n=50	4°C n=50
1	30	10	-	22	20	-	20
2	10	-	4	8	4	10	2
3	-	2	-	6	10	20	-
4	50	72	94	60	62	56	62
5	2	2	-	-	2	-	-
6	2	2	-	-	-	-	-
7	-	-	-	2	-	-	14
8	6	12	2	2	2	14	2

Colonies from milk products spoiled at 4° C and 20° C characterised and arranged into eight colony types

1. Gram negative rods, aerobic, oxidase positive and motile - *Pseudomonas*
2. Gram positive rods, aerobic, fermentative or giving no Hugh &Leifson reaction, motile, spore forming - *Bacillus*
3. Gram positive, anaerobic rods -*Lactobacillus*
4. Gram negative fermentative rods -Enterobacteriaceae
5. Gram negative, aerobic, oxidase negative - *Acinetobacter*
6. Gram positive, aerobic, non-motile cocci - *Micrococcus*
7. Gram positive, fermentative, non-motile cocci -*Staphylococcus*
8. Other organisms that gave variable or no reactions

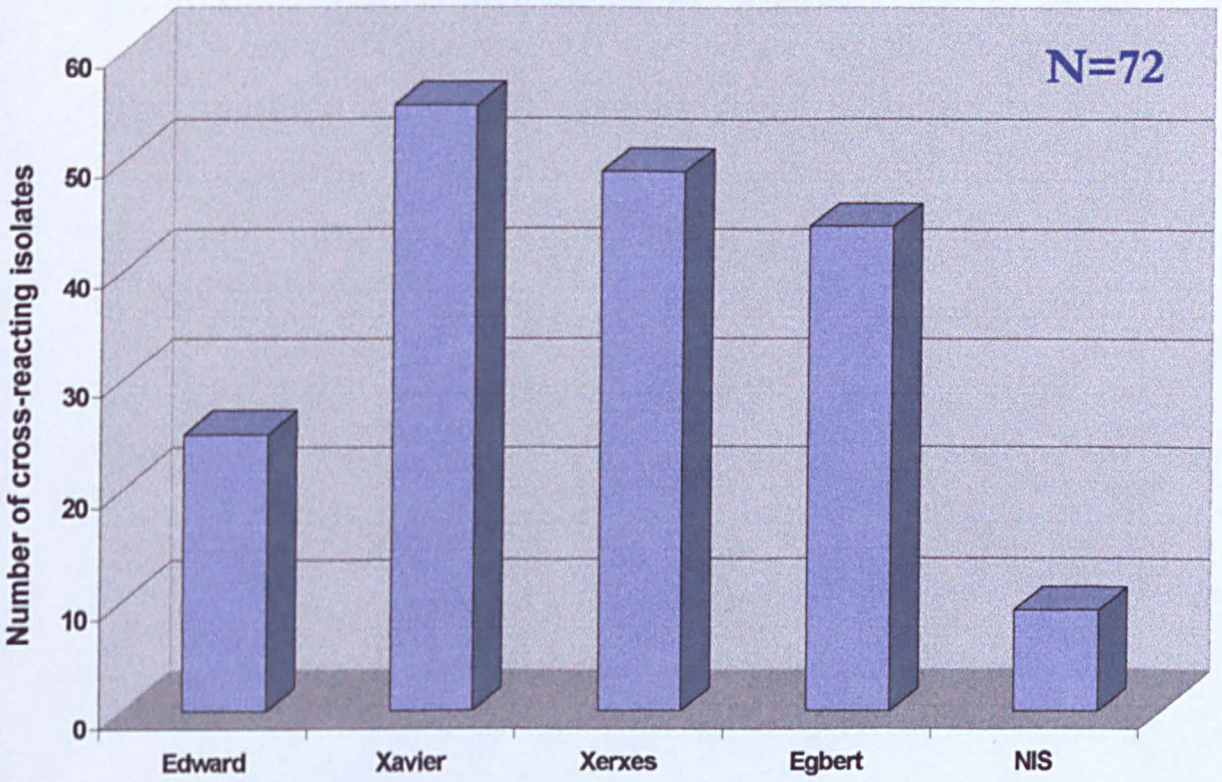
The identification of the organisms within each category was not confirmed and is given as examples of organisms that fit the category description.

TABLE 6.2 THE PERCENTAGE OF CHARACTERISED ORGANISMS FALLING WITHIN EIGHT DEFINED CATEGORIES

Product	Storage Temperature (°C)	Number of *Pseudomonas* isolates tested	Number of Proteolytic Isolates on Skim milk agar	
			4°C	30°C
Semi-skimmed	20	5	0	1
Semi-skimmed	4	25	0	0
Full fat	4	16	7	2
Single Cream	4	15	1	0
Raw	4	16	10	2

TABLE 6.3 THE PROTEOLYTIC ACTIVITY OF *PSEUDOMONAS* ISOLATES AT 4°C AND 30°C

Figure 6.6 Number of *Pseudomonas* isolates cross-reacting with each antiserum



Xavier (raised against *Ps. fluorescens*), Xerxes, and Egbert (both raised against *Ps. putida*) cross-reacted with 55, 49 and 44 isolates respectively. Edward (raised against *Ps. fluorescens*) cross-reacted with 22. None of the isolates tested that were obtained from spoiled milk products at 20°C (5 isolates) cross-reacted with Edward antiserum. Of the organisms that did cross-react with Edward antiserum 9 were from full fat milk, 9 were from skimmed milk and 4 from single cream all at 4°C.

In the presence of Xavier antiserum none of the spoilage organisms isolated at 20°C cross-reacted with it. The majority of raw milk isolates cross-reacted with Xavier antiserum (12/14). Of the 55 isolates obtained from psychrotrophic spoilage 40 cross-reacted with Xavier (~73%).

Of the 55 isolates obtained from psychrotrophically spoiled pasteurised milk products 23 of them did not cross-react with Egbert antiserum. None of the organisms (5) isolated at 20°C cross-reacted with Egbert antiserum. Full fat milk produced 6 cross-reactants, semi-skimmed milk produce 15 and single cream produced 2.

As with all the other antisera none of the five isolates retrieved from spoilage at 20°C cross-reacted with Xerxes antiserum. The spoilage isolates from raw milk all cross-reacted with Xerxes antiserum with the exception of two. The two exceptions were the same two that did not cross-react with Xavier antiserum.

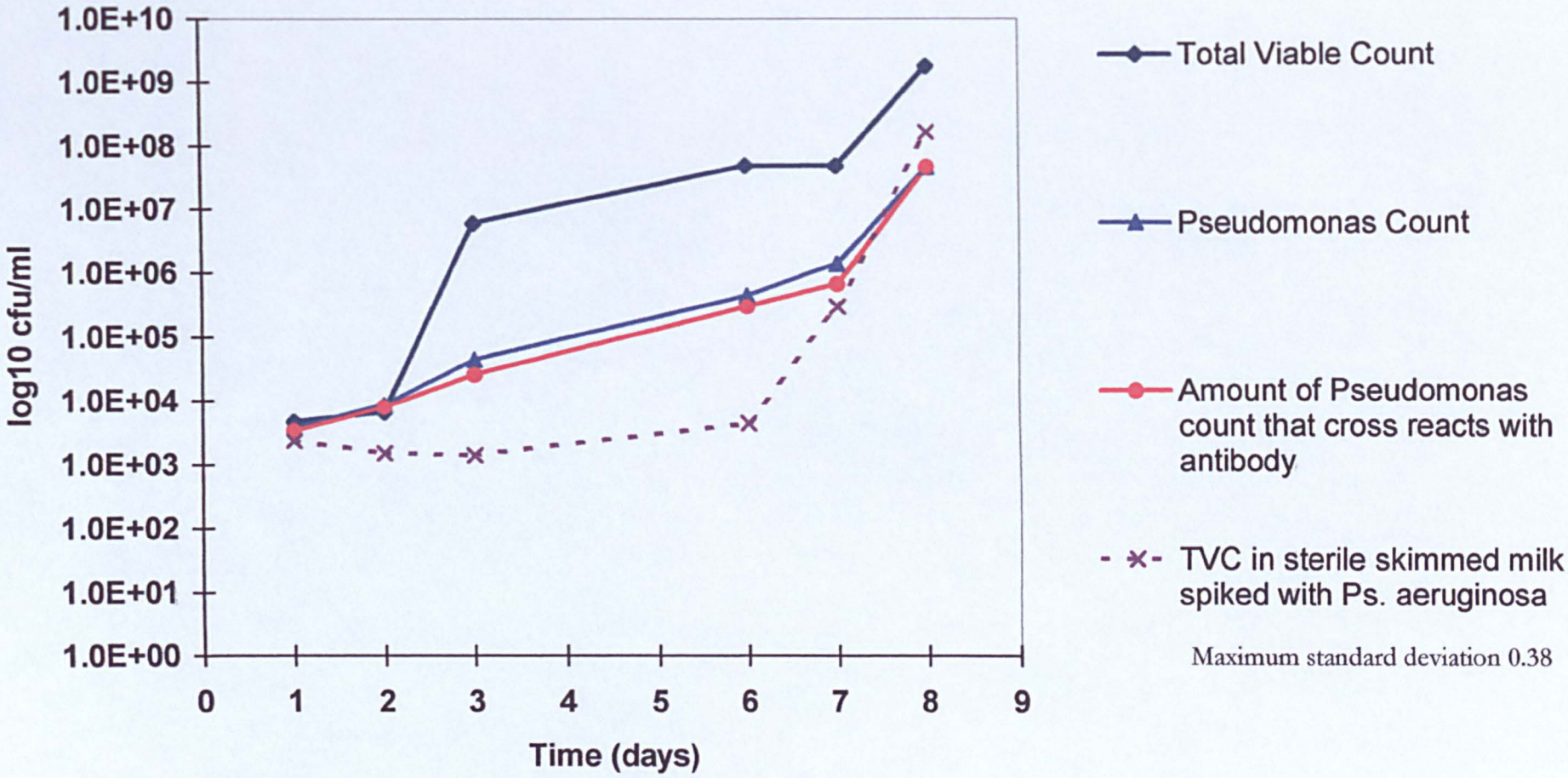
6.4.6 FOLLOWING THE GROWTH OF AN INTRODUCED ORGANISM IN SEMI-SKIMMED MILK USING ANTIBODIES

The growth of the introduced organism (*Ps.aeruginosa* from spoiled chicken) in semi-skimmed milk is shown in Figure 6.7. After day 2 the total microbial population rapidly outgrew the *Ps. aeruginosa* population identified with the antiserum. The *Ps. aeruginosa* population's growth curve closely mimicked the *Pseudomonas* count indicating that the majority of the *Pseudomonas* population comprised the introduced organism.

The population of the introduced organism had a long lag phase or slow exponential rate and towards the end of the spoilage period (day 7) began to grow rapidly. The total microbial population had a very short lag phase.

Growth of the *Ps. aeruginosa* introduced into sterile skimmed milk showed a long lag phase until day 6 which was then followed by rapid exponential growth attaining a final count of 7.1 x 10^8 cfu/ml by day 8.

Figure 6.7 Growth of Introduced *Ps. aeruginosa* in Pasteurised Semi-skimmed Milk



Maximum standard deviation 0.38

6.5 Discussion

The high total counts seen at the end of spoilage (~ 10^{11}cfu/ml) when following the natural spoilage of milk products at 4°C was influenced by the incubation time of 48 hours for the agar plates. The incubation time for the *Pseudomonas* CFC and nutrient agar was 24-48 and 24 hours respectively, however, 48 hours was used routinely for both types of agar to allow the psychrotrophic organisms time to grow to a reasonable colony size for colony lifts. However, cell numbers of the order of 10^{11} per ml are unfeasibly high for *Pseudomonas* cells. The maximum packing density of bacterial rods in $1cm^3$, with dimensions of 0.5µm by 1.7µm (similar to the dimensions of *Pseudomonas* cells) is 7.5 x 10^{11} and would represent solidly packed cells. The erroneously high cell numbers were possibly attained during the performance of the colony lift procedure. After the immuno-blotting of the colony-lifted membranes each of the spots counted may not have arisen from an individual colony e.g. due to splashing during the colony lifts.

Full fat milk was unfit for consumption by day 8 when stored at 4°C. At the time of spoilage a sour smell was associated with a total microbial count of 10^6 cfu/ml and a pH of 6.5. Cousin (1982) stated that flavour changes occur in pasteurised milk stored at refrigerated temperatures when the psychrotrophic count reaches 5 x 10^6 - 20 x 10^6 cfu/ml. At the time of sensory changes, semi-skimmed milk had a total viable count ten fold higher than that seen in full fat milk that may be due to the fact that the initial contamination levels were higher. Semi-skimmed milk is more highly processed than full fat milk and contamination events may be more frequent. The pattern of spoilage in the full fat milk and semi-skimmed milk were very similar. The green coloration observed in full fat and semi-skimmed milk probably resulted from the production of fluorescent pigments commonly associated with *Ps. fluorescens* (and other *Pseudomonas* species). The granular texture observed in semi-skimmed milk was possibly due to the

presence of *Bacillus* spp. *Bacillus* can produce lecithinase that hydrolyse lecithins present in the milk fat globule membrane leading to an aggregation of the fat (Cousin, 1982; Thompson, 1996). The aggregation of the fat is known as the "bitty cream effect". The difference in composition of the two products (e.g. fat content) had no affect on growth. Fat contents as low as those found in milk will have little affect on the structure of the heterogeneous oil-in-water emulsion and microbial growth would be similar to that found in a non-structured liquid (Brocklehurst *et al.*, 1995).

The psychrotrophic spoilage of pasteurised full fat and semi-skimmed milk was mediated by *Pseudomonas* species. The fact that *Pseudomonas* spoils refrigerated milk can be found in any textbook. What is noteworthy is how well the *Ps. fluorescens* antibody followed the spoilage population and reflected the growth pattern of the dominant species (Figures 6.2 & 6.3). The antiserum chosen to follow the spoilage of milk products was raised against *Ps.fluorescens* isolated from raw milk at 4°C. The antibody population within the antiserum recognised all (as compared to the total viable count) of the spoilage *Pseudomonas* population of pasteurised full fat and semi-skimmed milk.

Ternstrom *et al.* (1993) characterised the spoilage flora of raw and pasteurised milk and found that the spoilage flora at 5°C comprised *Bacillus polymyxa* (26%), *Ps. fluorescens* biovar 1 (22%), *Ps. fluorescens* of other biovars (6%), *Ps. lundensis* (6%), Enterobacteriaceae (6%), *Sphingobacterium-Flavobacterium* spp., *Acinetobacter* (4%), *Bacillus* spp. (4%), *Ps. putida* (2%), *Leuconostoc* spp. (2%) and *Lactobacillus* spp. (1%). The antiserum used to track spoilage *Ps.fluorescens* of the milk products may also be detecting Ps. *lundensis* and *Ps. putida*. *Ps. fragi* (classified as a biovar of *Ps. fluorescens*) and *Ps. aeruginosa* may also be encountered. Previous characterisation of the Xavier antiserum indicated that the antibody population would cross-react with *Ps. fluorescens* biovars and *Ps. putida*.

Single cream was unfit for consumption by day 4 as the product thickened and produced a sour smell. Souring occurred when the microbial population reached 5.3 $x10^5$ cfu/ml with an associated pH of 6.5. The pH fell at day 8 and coincided with a plateau in the *Pseudomonas* count and a fall in the number of cross-reacting *Pseudomonas* species (Fig.6.4). After two days in a reducing pH environment the total *Pseudomonas* population recovered to dominate the microbial flora by day 12. However, the population of *Pseudomonas* cross-reacting with antiserum did not adapt as quickly as the total *Pseudomonas* population. A group of *Pseudomonas* species that could tolerate the reducing pH seemed to have succeeded the *Pseudomonas* sub-population that could not tolerate the changing pH. The *Pseudomonas* species that could tolerate the pH did not cross-react with the antiserum. Alternatively, due to the falling pH the *Pseudomonas* cells could have altered phenotypically such that the antibody-binding sites on the surface of the cells could have been obscured or changed. The possible change in antibody-binding sites on the cell surface would lead to a decrease in the number of *Pseudomonas* cross-reacting with the antiserum as seen with single cream.

The growth of the spoilage population within raw milk had a short lag phase due to the high numbers of the initial diverse microflora (Cousin, 1982: Doyle *et al.*, 1997). The tracking of the *Pseudomonas* population in raw milk demonstrated that *Pseudomonas* was initially present in fairly large numbers amongst other dominant genus such as *Bacillus* and Enterobacteriaceae. After three days *Pseudomonas* became the dominant genus. The *Pseudomonas* population cross-reacting with the antiserum did not closely track the *Pseudomonas* count as seen with full fat milk. In a declining pH environment the number of *Ps.fluorescens* cross-reacting with the antiserum fell in a similar manner to that seen with single cream. The fall in pH seen in raw milk was more rapid than the fall seen in semi-skimmed milk. The effect of a declining pH environment in conjunction with the presence of a mixed microbial population possibly dominated by *Bacillus* spp. led to the reduction in the number of antiserum cross-reacting *Pseudomonas*. Further work would be needed to clarify the growth of the cross-reacting *Pseudomonas* population during the

early stages of spoilage. A likely scenario is that the cross-reacting *Pseudomonas* population would follow the *Pseudomonas* count closely and at day 4, when the pH falls, the cross-reacting *Pseudomonas* population numbers would fall also.

As mentioned previously, Ternstrom *et al.* (1993) found that Enterobacteriaceae made up 6 % of the microflora of refrigerated pasteurised milk. Characterisation of members of the microbial flora with differing morphologies led to a high proportion of isolates being categorised as colony type 4 (Enterobacteriaceae), well beyond 6%. The percentage of the flora falling into each category does not relate to the percentage of each genus within the total microflora. Identifying a range of morphologies gave an indication of the diversity of the microflora present. The fact that colony type four organisms were the most commonly isolated organism was possibly due to there being a wide variation in colonial morphology of these organisms. Those colonies exhibiting different morphologies were selected for and hence, the selection process may have been biased towards cell type four isolates. In contrast *Pseudomonas* colonies, although more numerous, were more uniform in morphology and therefore fewer types examined.

The flora of refrigerated milk products was dominated by psychrotrophic bacteria that are associated with proteolytic and lipolytic exoenzymes that degrade milk protein and fat. The proteolytic assay of *Pseudomonas* species isolated from psychrotrophically spoiled milk showed that all the isolates were not proteolytic at 4°C or 30°C. In the case of semi-skimmed milk none of the 25 isolates were proteolytic on skim milk agar at either 4°C or 30°C. Philips *et al.* (1981) reported that not all of the proteolytic bacteria found in milk were actively degradative, which supports the findings present here.

On the introduction of *Ps. aeruginosa* to semi-skimmed milk (figure 6.7) the *Pseudomonas* cross-reacting population was dominant for the first two days but their dominance in the flora decreased between day 3 & 7. The dominance of the *Pseudomonas* cross-reacting

population was re-established on day 8. The variation in prominence of the introduced organism within the total microbial flora suggests that bacteria introduced into food systems do not grow in the same manner as the existing microbial flora until a period of adaptation has taken place. Hence, studies that use introduced organisms in foods to reflect bacterial growth of members of the natural population should be treated with caution.

The spoilage of double cream was investigated to elucidate how the structure would affect the growth of the spoilage flora. However, the viscosity and opacity of the product at times made enumeration and colony lifts difficult to achieve.

Non-specific binding can be problematic with antibody based assays but can be estimated with NIS controls. Additionally, a visual assessment of the colour of a spot on a nitrocellulose filter can be subjective due to the wide variation in the colour of the spots seen (Chapter 3). The development of image analysis software facilitates the comparisons of the colour intensity of the spots on a nitrocellulose filter in a rapid, quantifiable and objective manner. Overall, the use of antibody-linked staining can be a very reliable and specific way to follow sub-populations within a complex mixed flora.

7 *IN SITU* DETECTION WITHIN A FOOD MODEL

7.1 INTRODUCTION

The safety and quality of foods depends essentially on the degree to which they support microbial growth. The structure of a food can affect microbial growth (Dodd & Waites, 1992; Robins & Wilson, 1994). Most dairy products, meat pastes, gels and multi-component convenience foods have highly complex physical structures. Traditionally, studies of the growth of micro-organisms in food have been implemented using model-based homogenous aqueous systems disregarding any implications that the food structure may impart. The development of a model system that incorporates the structure of commonly found foods could be the basis of a more accurate protocol for predicting microbial growth in foods.

The heterogeneity of components and available nutrients within most foods leads to a series of micro-environments that vary in their ability to support microbial growth. An investigation of the sites of microbial growth, together with the specific bacteria that occupy them, within foods would provide valuable information regarding the conditions *in situ* that limit growth, information which liquid systems could not yield (Dodd & Waites, 1991). This information could lead to the application of a reduced and highly focused preservative and additive regime during food processing.

Antibody-linked probes are highly compatible with locational studies in food (Alcock *et al.*, 1992; Dodd & Dainty, 1992; Stringer *et al.*, 1995a). In addition to the detection of microbes, microbial products and food components can also be detected (Stringer *et al.*, 1995a; Stringer *et al.*, 1995b). Antibodies can be labelled, directly or indirectly, with various enzymic or fluorescent markers that are compatible with various differential-staining techniques.

7.1.1 EMULSIONS

Most processed foods (butter, margarine, dips, gravies, sauces, cake batters, cream liqueurs and coffee whitener) consist partly or wholly as emulsions. An emulsion is a dispersion of two immiscible liquids, one of which is finely divided and uniformly distributed as droplets (the dispersed phase) throughout the other (continuous phase). An alternative definition is that they are thermodynamically unstable heterogeneous mixtures that can be stabilised by a third phase, an emulsifying agent.

When two immiscible liquids are in contact with each other, they will tend to maintain as small an interface as possible. Consequently, it is difficult to mix the two liquids. When shaken together, spherical droplets will form, and an interfacial tension will be maintained between the two liquids. With the inclusion of surface-active molecules, which will orient between the two phases, the interfacial tension will be reduced (Figure 7.1). This will result in miscibility of the two liquids. The surfactant may also form a rigid interfacial film (which acts as a mechanical barrier to coalescence of the globules) or an electrical double layer (which results in repulsive electrical forces between approaching droplets to minimise coalescence).

There are two types of simple emulsions, oil-in-water and water-in-oil. Multiple emulsions can be prepared by emulsifying an emulsion into another external phase (Figure 7.2).

The aqueous phase within an emulsion is the site of microbial growth (Tuynenburg Muys, 1971). In an oil-in-water emulsion, such as dairy cream, the interstices between the oil droplets are of the same order as the diameter of the droplets and dependent on the droplet concentration (Brocklehurst *et al.*, 1995). The dimensions of bacterial rods are of the order of 0.5 - 5 μM and the space available for growth within a closed packed emulsion can be limited. Hence the microscopic structure can influence the growth of the bacteria within the continuous phase (Brocklehurst *et al.*, 1995).

To create an emulsion, energy needs to be applied, mechanical and/or chemical. Violent mixing or shearing that might disperse the oil phase into minute droplets creates mechanical emulsions. In time the mechanical emulsion will break down without additional treatment. An emulsion is stabilised with the inclusion of an emulsifier or surfactant.

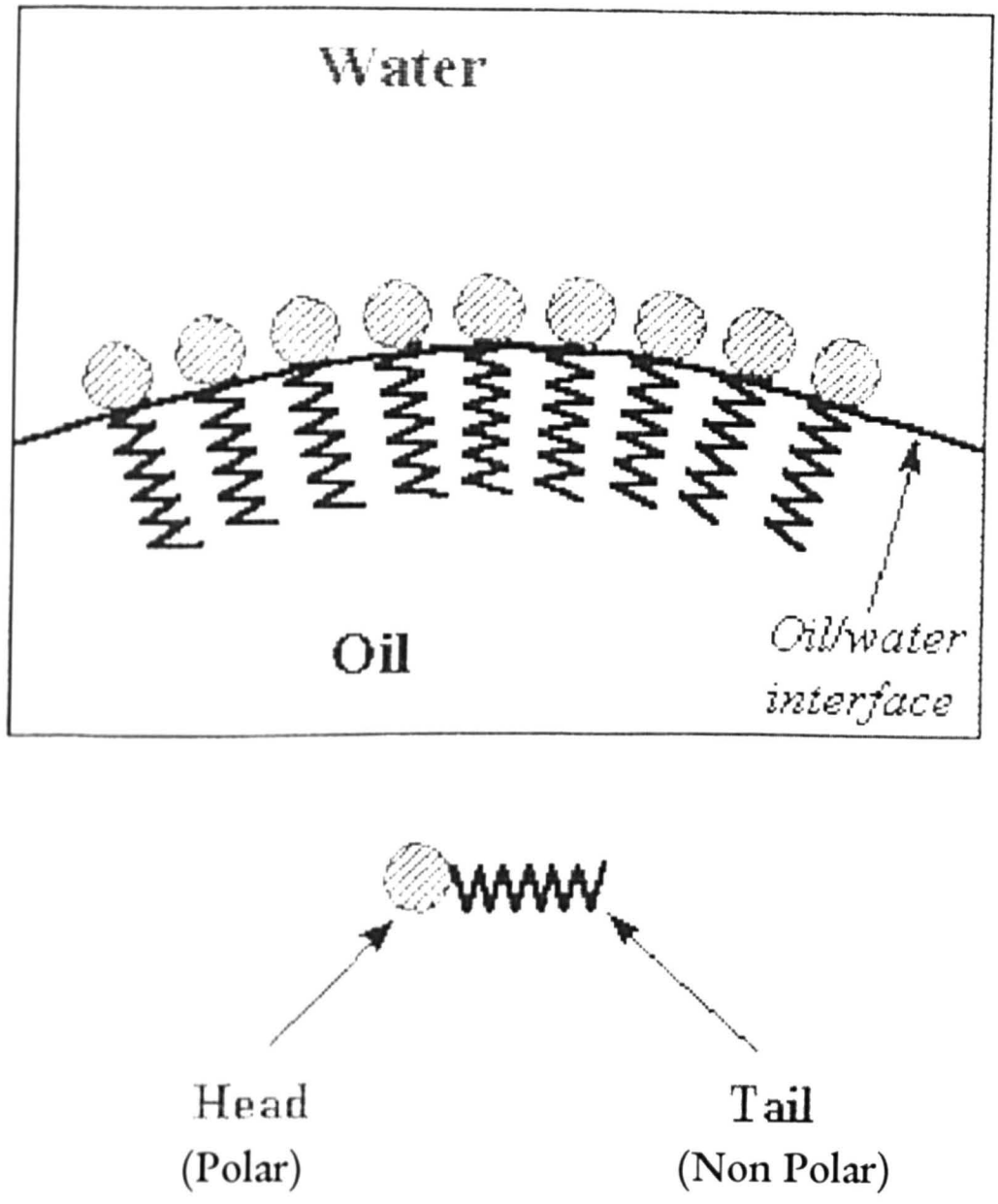


Figure 7.1 Diagram showing the emulsifying agent or surfactant at the oil/water interface.

The surfactant has lipophilic and hydrophilic structural portions within their molecular structures.

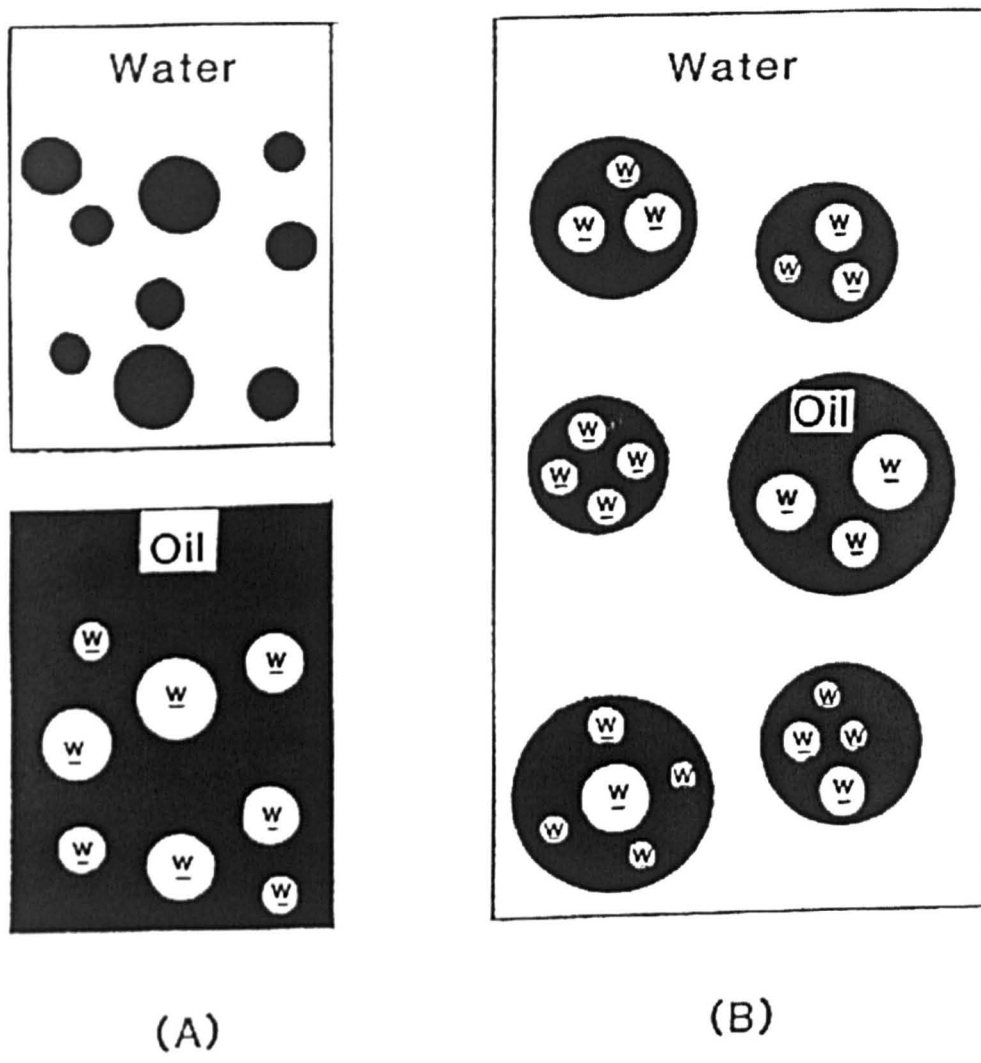


Figure 7.2 (A) Simple two phase emulsion; top - oil droplets in water (o/w); bottom - water droplets in oil (w/o). (B) Multiple emulsion, water-in-oil-in-water emulsion (w/o/w).

Based on diagram from Food Emulsions, 1997 Eds. Stig. E. Friberg and Kare Larsson Pub. Marcel Dekker Inc.

7.1.2 FOOD EMULSION MODELS

In 1991 the Institute of Food Research, Norwich initiated a research programme on the physicochemical principles underlying the growth of micro-organisms in complex foods (Robins *et al.*, 1994). From this work was developed several food models, for instance gels (where bacteria are immobilised within a uniform medium) and water-in-oil and oil-in-water emulsions (where bacteria grow in proximity to oil droplets of a comparable size to themselves).

Using the water-in-oil emulsion model it was shown that the concentrations of the micro-organism within droplets of the aqueous phase could be higher than that usually found in liquid-grown cultures. This was due to the utilisation of dissolved oxygen in the oil phase (Robins *et al.*, 1994). The enhanced oxygen conditions also resulted in an increased survival time of the bacteria (Gunning, 1996). In contrast to this, bacteria growing in the continuous (aqueous) phase in oil-in-water emulsions were constrained to grow as colonies and hence grew at a slower rate than that of the equivalent aqueous grown cells (Brocklehurst *et al.*, 1995).

The model oil-in-water emulsions described above comprised of oil (non-food grade), surfactant (non-food grade) and microbial liquid culture media (non-food). Natural oils used in foods contain a number of impurities, which makes the formation of stable emulsions difficult. The effectiveness of non-food grade surfactants (e.g. Tween) to produce stable emulsions is also limited. The composition of the aqueous phase may again affect stability. The closer a model system reflects real food the more relevant the information or predictions yielded from it will be.

7.2 AIMS AND OBJECTIVES

The aims were to develop a near food grade oil-in-water cream emulsion model and to use labelled antibodies to locate specific bacteria within the model.

Why use a model system to examine growth in this food type? Cream emulsions vary from sample to sample and are innately unstable eventually leading to droplet flocculation (moving together), coalescence (merging) or creaming (oil rising) events. Such physical changes may lead to changes in the emulsion structure and hence, changes in bacterial growth. A model emulsion was developed to allow growth to be studied under standardised conditions. This allows *Pseudomonas* to be studied under more controlled

conditions. A model would also enable the study of known bacteria without the presence of a natural flora of unknown composition. Additionally, dairy cream and milk products contain proteins that auto-fluoresce which would mask the fluorescently labelled antibodies used to locate specific bacteria. However, a model emulsion could be manipulated to reduce auto-fluorescence.

7.3 METHODS

7.3.1 PREPARATION OF INOCULA

Pseudomonas aeruginosa (NO31), *Ps. fluorescens* (EM1), *Ps. putida* (EM12) and *E. coli* (Ej1a) were prepared as per the method of Brocklehurst *et al.* (1995). Model emulsions were made that were inoculated with a single isolate or mixed culture emulsions were made that were inoculated with two isolates.

7.3.2 MODEL OIL-IN-WATER EMULSION

Model emulsions were made aseptically in a stainless steel Waring blender (Fisons Scientific Equipment, UK). Sterile hexadecane (42 ml, Aldrich Chemical Co. Ltd.) was emulsified with UHT skimmed milk (100 ml, bought locally) and a non-ionic surfactant Brij 25 (polyoxyethylene lauryl ether, 0.5%v/v, Pierce and Warriner, Chester, UK) using a fixed shear cycle. The emulsions were inoculated to give a concentration of viable bacteria of 100/ml continuous phase. Non-inoculated emulsions were used as controls and for droplet size determinations.

Aliquots of inoculated and control emulsions were centrifuged (3000g for 20 minutes at 25°C) in order to produce a cream layer that was separated from the residual continuous phase. Control emulsions were used for droplet size determinations.

The creamed emulsions were aseptically transferred to sterile 5 ml syringes, the needle end of which had been removed leaving an open tube with plunger. Once filled with emulsion the ends of the syringes were covered with Saran wrap. The emulsions were then incubated upright (25°C).

7.3.2.1 Shearing protocol

The oil, milk and surfactant were placed into a sterile Waring blender and emulsified using the following shear cycle.

1. 30 seconds low speed, 30 seconds stop
2. 30 seconds low speed, 30 seconds stop
3. 30 seconds high speed, 30 seconds stop
4. 30 seconds high speed, stop

The shear protocol yielded dispersed oil-in-water emulsions of 30% (before centrifugation) or 70% (v/v) (after centrifugation) (Brocklehurst *et al.*, 1995). Tim Brocklehurst at the Institute of Food Research, Norwich, is acknowledged for the determination of the oil concentration in these emulsions.

7.3.3 PHYSICAL CHARACTERISATION OF THE OIL-IN-WATER EMULSION

The droplet size distribution was determined during incubation (25°C) using a Coulter LS Particle Analyzer. The emulsion was considered stable if a consistent droplet size distribution was maintained over three days.

7.3.4 DETERMINATION OF GROWTH RATE

Pasteurised whipping cream (bought locally, fat content ~ 35%) and 30% oil-in-water model emulsion were inoculated with *Ps. fluorescens* (EM1, $2x10^2$ cfu/ml) and incubated at 25°C. At intervals aliquots were diluted in buffered peptone water and plated in duplicate onto tryptone soya agar. Aliquots of uninoculated pasteurised whipping cream and 30% emulsion were used as controls.

7.3.5 LABELLED ANTIBODIES

Antisera raised against the *Pseudomonas* isolates used were purified (see section 2.13) and conjugated to either FITC (fluorescein isothiocyanate, section 2.15.2) that fluoresces green or biotin (section 2.15.1). Staining of bacterial cells was done directly with FITC labelled antibodies or indirectly with phycoethryin conjugated to avidin (avidin has a very high affinity to biotin), which fluoresces red, or FITC goat anti-rabbit antibodies.

7.3.6 ANTIBODY STAINING

The FITC labelled antibody (1:20 to 1:40 dilution) was applied to the air dried colonies. The biotinylated antibodies were incubated with the avidin conjugated fluorochrome (30 min at 30°C, as per the manufacturer's instructions) before application of the stained

preparation to the microscope slide. The appropriate primary antibody was incubated with the FITC labelled secondary antibody (30 min, 30°C in the dark) before application to the sample. The slides were then incubated (20-30 min at 30°C in the dark), gently rinsed in mounting solution (poly vinyl alcohol, Sigma) and viewed under the epifluorescent microscope.

7.3.7 MICROSCOPY

The basic structure of the emulsion was observed by using light (samples stained with crystal violet or toluidine blue) and differential interference contrast microscopy.

The bacterial growth form within the 70% oil-in-water emulsion was observed after the oil phase was removed. This was achieved by placing portions of the emulsion onto the surface of warmed chloroform: methanol (1:1) solution and allowing the bacterial colonies to fall to the bottom of the vessel. Colonies were removed from the bottom of chloroform: methanol solution with a pipette onto a microscope slide coated with poly l lysine (Sigma). The method was taken from Parker *et al.* (1995). Epifluorescent microscopy was conducted using a Leitz Ortholux II microscope to view the fluorescently stained colonies. A mounting solution of tris buffered polyvinyl alcohol (Sigma) was used to preserve colour

7.4 RESULTS

7.4.1 PHYSICAL CHARACTERISATION OF THE OIL-IN-WATER EMULSION

The distribution of the droplet sizes for the 30% and the 70% emulsion was the same. A typical droplet size distribution can be seen in Fig. 7.3. The mean droplet size was approximately 2.7μm and remained constant for three days (+/- 0.1μm).

7.4.2 RATE OF BACTERIAL GROWTH IN REAL AND MODEL EMULSION

The rate of growth of *Ps.fluorescens* in a 30% model emulsion and real cream can be seen in Fig. 7.4. The growth rates in both systems were very similar to each other and to the rate of growth seen in liquid microbiological media (data not shown).

7.4.3 MICROSCOPY

The basic structure of the 30% and 70% model emulsion can be seen in Figure 7.3. The 30% emulsion was very fluid, with large distances between oil droplets. The 70% emulsion was very thick (similar to mayonnaise) and the oil droplets were tightly packed.

A sample of *Ps. fluorescens*-inoculated 70% emulsion stained with acridine orange can be seen in Picture 7.1. Viable cells appear red and dead cells green. The original position of the oil droplets can be clearly seen.

Pictures 7.2 and 7.3 were taken from the same creamed emulsion sample that was inoculated with *Ps. fluorescens* (for 2 days) and indirectly antibody stained with phycoethryin.

Picture 7.2 shows the growth of cells in the interstices between the oil droplets. Picture 7.3 shows a very large colony. Behind the colony a large droplet of oil can just be detected. The presence of many indentations on the surface of the colony implies the proximity of many other smaller droplets during growth.

A 70 % emulsion was made that was inoculated with both *Ps. aeruginosa* and *Ps. fluorescens*. (1:1). The *Ps. fluorescens* was indirectly labelled with phycoethryin and the *Ps. aeruginosa* cells were directly labelled with FITC (Picture 7.4). The *Ps. fluorescens* cells have been attracted to the surface of the oil droplets and are engulfing it. The background is green due to the presence of FITC. No *Ps. aeruginosa* cells were detected. This could be due to inadequate staining of the *Ps. aeruginosa* cells or that under the prevailing culture conditions the *Ps. fluorescens* cells outgrew the *Ps. aeruginosa* cells. To confirm the latter, colony lifts from viable count plates could have been conducted and the membranes immunoblotted with specific antisera to each species.

Another mixed-culture 70% emulsion was made using *Ps. fluorescens* and *E. coli*. (1:1). The *Ps. fluorescens* cells were indirectly stained with FITC goat anti-rabbit antibodies. The *E.coli* cells were unstained. Picture 7.5 shows the labelled *Ps. fluorescens* cells within small colonies after incubation for one day.

Additional samples were taken from the same mixed culture 70% emulsion as seen in Picture 7.5. The samples were placed directly onto a microscope slide (without the oil being removed). One sample was stained with crystal violet and viewed under a light microscope (Picture 7.6 A) and another sample was indirectly antibody stained with FITC goat anti-rabbit antibodies (after the incubation step during antibody staining the slide was not rinsed for fear of washing the sample away: Picture 7.6B). The density of cells seen in Picture 7.6B is less than that seen in Picture 7.6A, as expected.

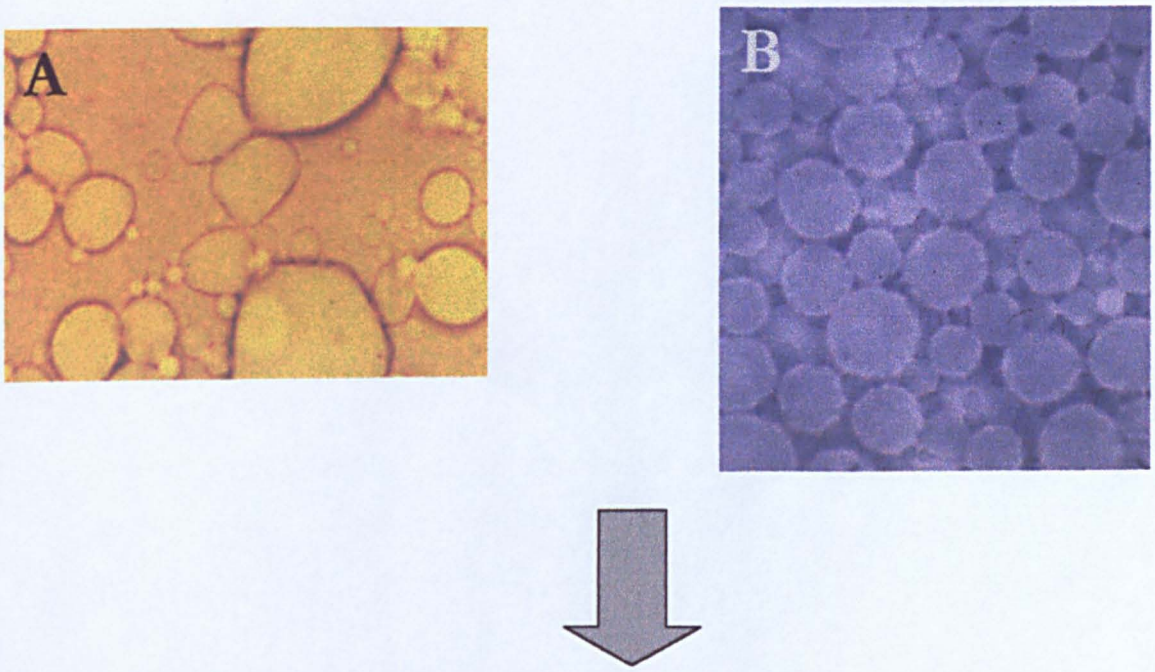
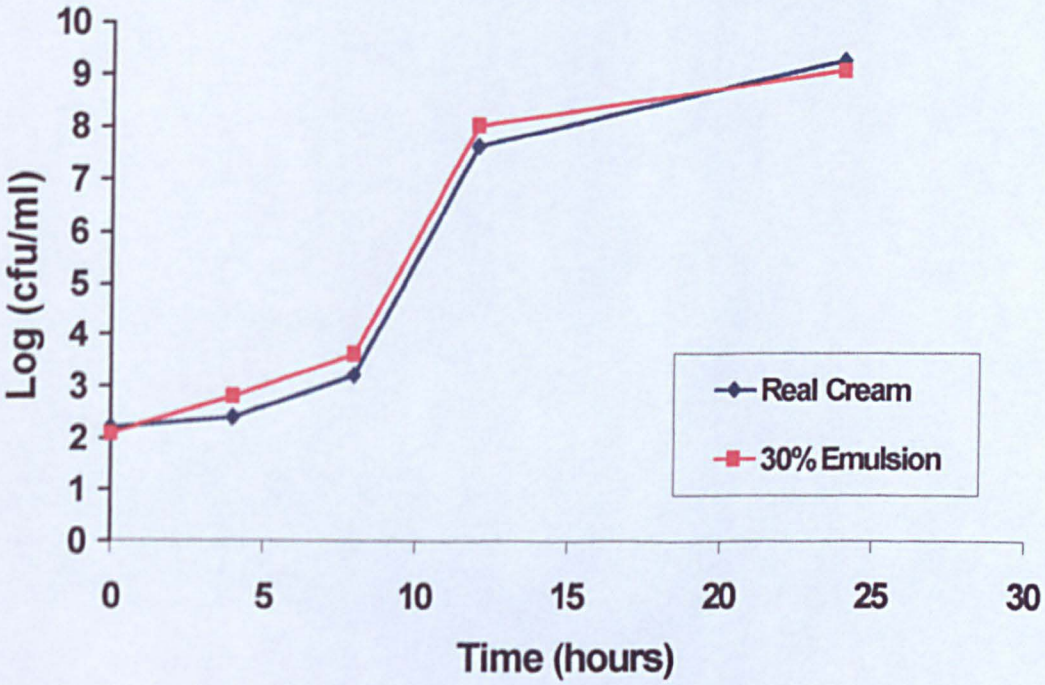


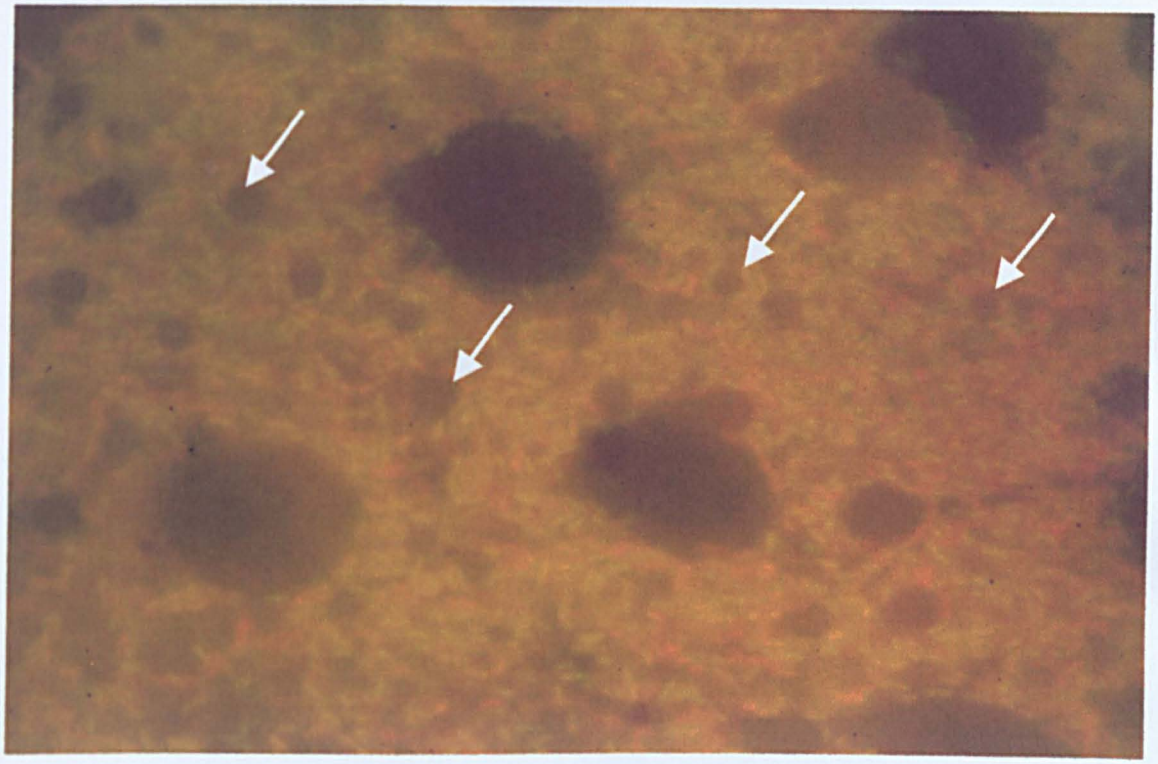
Figure 7.3 Structure of the near-food-grade model emulsion

A: 30% model emulsion viewed under a light microscope after one day. B: Model 70% emulsion stained with crystal violet and viewed under a light microscope after one day. C: Histogram (produced by the Coulter LS Particle Analyzer) shows a typical distribution of oil droplet sizes within both model emulsions after one day.

Fig. 7.4 Growth of *Pseudomonas fluorescens* in cream and 30% emulsion model at 25C



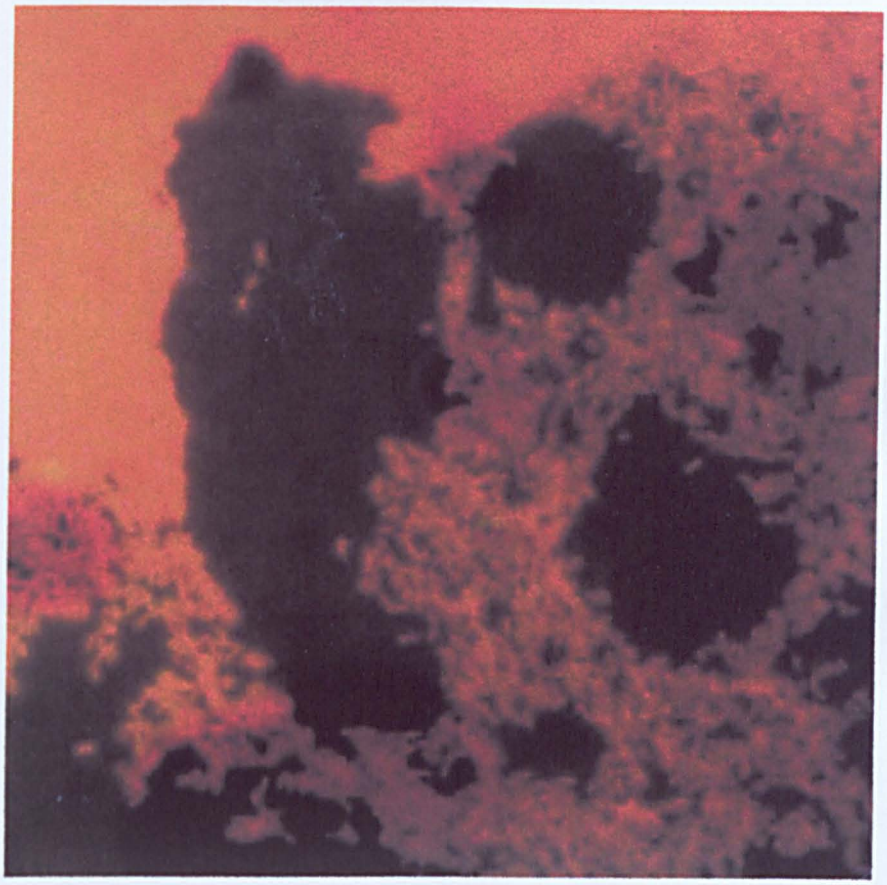
Standard deviation no greater than 0.41 cfu/ml at any point using data from two replicate experiments



PICTURE 7.1 A sample of near food grade model 70% oil-in-water emulsion inoculated with *Ps. fluorescens* (RM1) stained with acridine orange after 1 day.

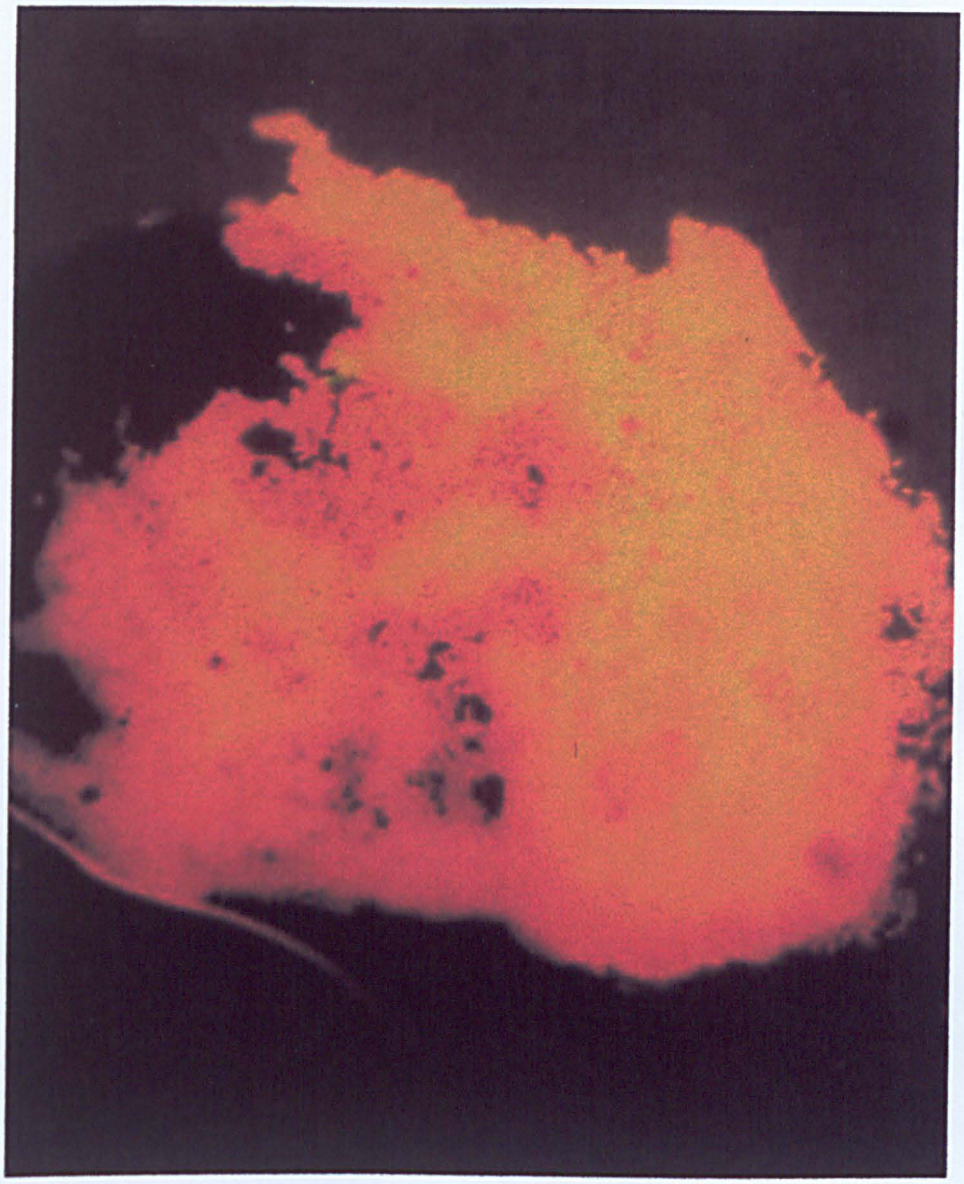
The arrows indicate the original location of some of the oil droplets.

Observed at a magnification x 1000



PICTURE 7.2 ***Pseudomonas fluorescens*** **(RM1) cells, indirectly labelled with phycoethryin conjugated with avidin, grown in a near food grade model 70% oil-in-water emulsion for 2 days.**

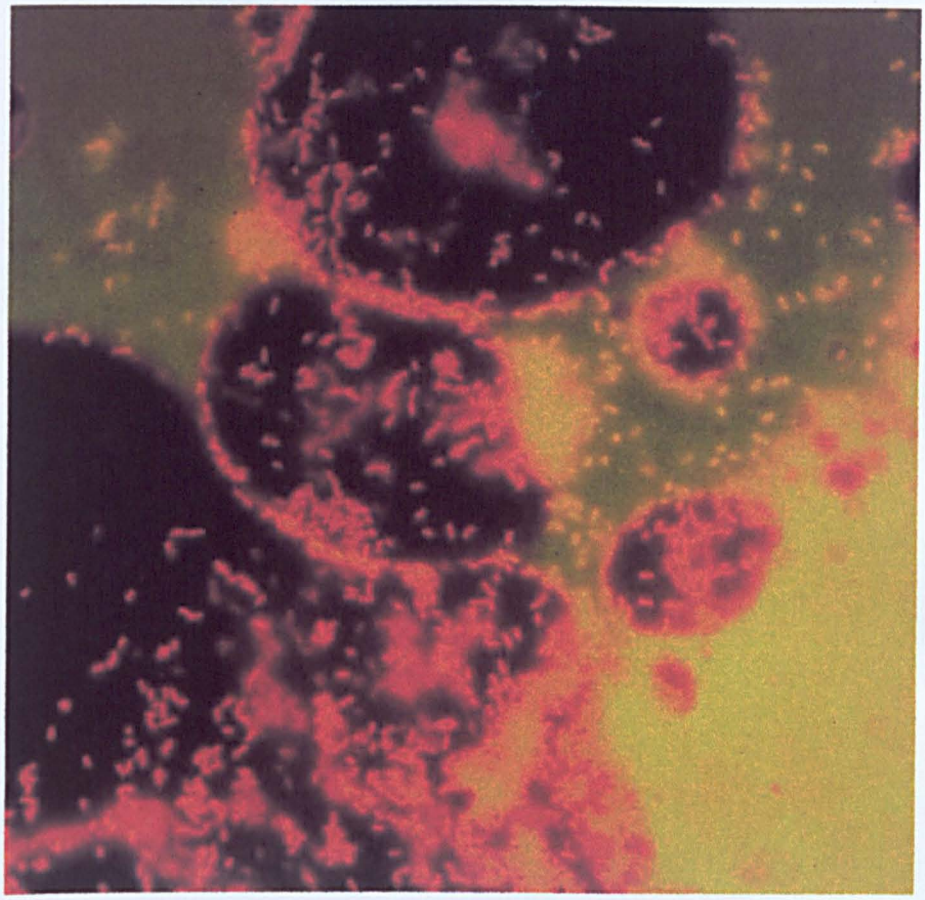
Observed at a magnification x 1000



PICTURE 7.3 A 2 day old colony of *Ps. fluorescens* (RM1) that was extracted from a near-food-grade 70% oil-in-water model emulsion.

The colony was indirectly labelled with phycoethryin conjugated to avidin.

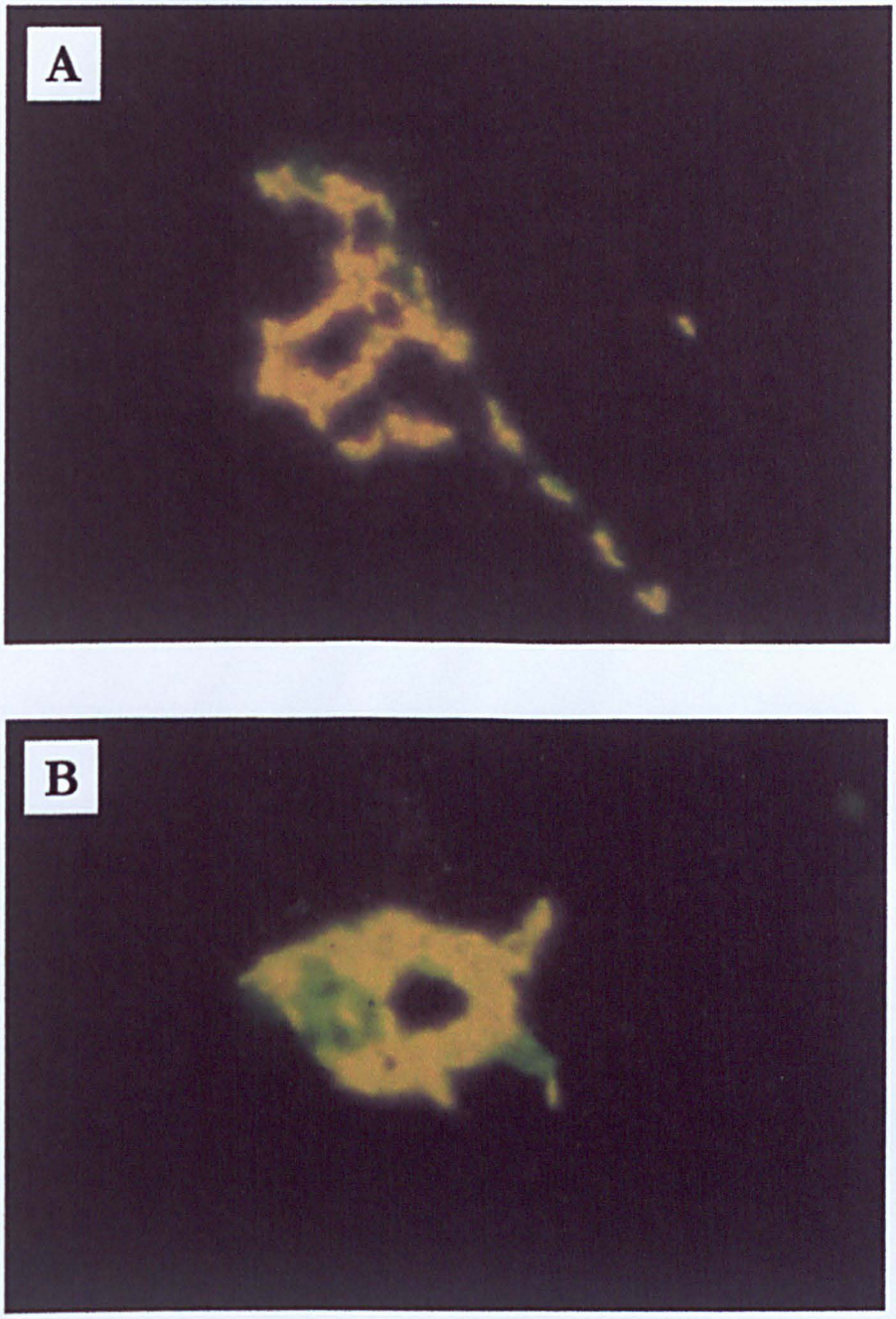
Observed at a magnification x 1000



PICTURE 7. 4 *Ps. fluorescens* (RM1) was grown in the presence of *Ps. aeruginosa* (NO31) in a 70% near food grade model emulsion for 1 day.

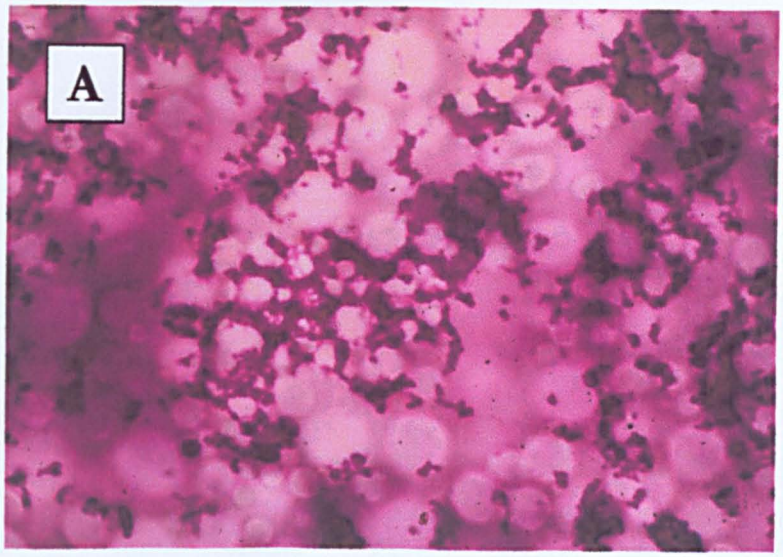
The *Ps. fluorescens* cells were indirectly labelled with phycoethryin conjugated to avidin and the *Ps. aeruginosa* cells were directly labelled with FITC. No *Ps. aeruginosa cells* can be seen. The *Ps. fluorescens* cells are attracted to the oil/water interface and the bacterial cells are beginning to envelop the oil droplets.

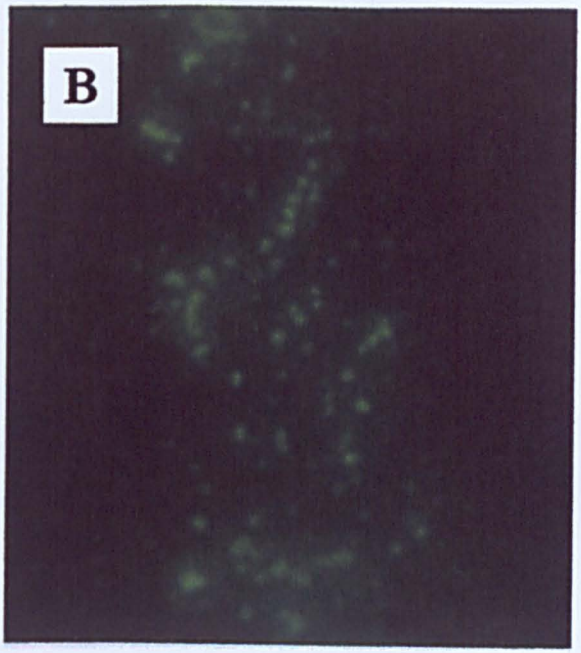
Observed at a magnification x 1000



PICTURE 7.5 A & B *Pseudomonas fluorescens* (RM1) colonies, indirectly labelled with FITC goat anti-rabbit antibodies, grown in the presence of *E. coli* (Ejla) within a 70% oil-in-water near food grade model emulsion.

Observed at a magnification x 1000





PICTURE 7. 6 70% near-food-grade model emulsion inoculated with *Ps.fluorescens* (RM1) and *E.coli* (Ej1a)

A: A 1 day old 70% near-food-grade model emulsion sample stained with crystal violet viewed under a light microscope. The emulsion was inoculated with both *Ps. fluorescens* (RM1) and *E. coli* (Ej1a). Observed at a magnification x 1000

B: The same model emulsion sample with the *Ps. fluorescens* cells indirectly labelled with FITC goat anti-rabbit antibodies. The density of the cells in B is much less than the density of the cells in A. Observed at a magnification x 1000

7.5 DISCUSSION

The near food grade oil-in-water emulsion was proved to be stable with a similar droplet size distribution as the oil-in-water model made by Parker *et al.* (1995, Figure 7.3). The incorporation of UHT skimmed milk into the model emulsion is an incremental improvement on the previous model. The ultimate goal would be to produce a stable model containing all food grade components.

The rate of growth of *Ps. fluorescens* was not affected by any of the components of the near food grade model as the rate of growth was similar to that found in a real food system with a similar oil content. At 30-35% oil content the emulsions are liquid and bacterial growth will be comparable to that of an aqueous solution as found by Parker *et al.* (1995). The form of growth of bacteria within the near food grade 70 % oil-in-water model emulsion was similar to that found by Parker *et al.* (1995) as seen in Pictures 7.1 - 7.4. The antibody stained *Ps. fluorescens* cells in Picture 7.4 clearly showed oil droplets with attracted bacterial cells surrounding them. The attraction of bacterial cells to the oil/water interface, due to the high oxygen tension at the interface, has been reported by other workers e.g. Robins *et al.* (1994) and Gunning (1996).

The antibody-linked staining tools developed specifically detected *Pseudomonas* within the near food grade model in pure (Picture 7.2 & 7.3) and mixed emulsion (Picture 7.5). Further work is needed to optimise the fluorescently labelled antibody protocols to simultaneously detect two different bacteria within the same model emulsion. This would facilitate investigations into the possibility of multixenic colonies, which may arise if two single bacteria of different origins were located adjacent to each other surrounded by oil droplets. The formation of such colonies would have far reaching food safety implications if pathogens could remain viable within a large colony consisting mainly of harmless or spoilage flora.

The fact that two organisms can co-exist closely within the emulsion was demonstrated in Picture 7.6. Within the mixed culture 70% emulsion antibody stained *Ps. fluorescens* was seen under fluorescent microscopy, the *E.coli* cells were unstained and therefore were not visible. The total density of the cells detected was much reduced compared to the same emulsion that was crystal violet stained, in which both *Ps. fluorescens* and *E.coli* cells were seen. The

reduction of cell density was probably due to the presence of the unlabelled *E.coli* cells. Confirmation of the change in density could be achieved by using image analysis such that the area occupied by the visible bacteria within the two differently stained emulsions could be compared per unit area. However, at the time of conducting these experiments image analysis equipment was unavailable.

Antibody staining of specific bacteria *in situ* was successfully achieved within the food model in the presence or absence of the oil phase. The removal of the oil phase allowed closer study of the formation of colonies within the model.

Chapter 8

DISCUSSION

Classification, nomenclature, and identification are three separate but interrelated areas of taxonomy (Staley & Krieg, 1984). Classification is the arrangement of organisms into taxonomic groups. Nomenclature is the assignment of names to the taxonomic groups according to international rules. Identification is the process of determining that a new isolate belongs to one of the established taxa (Staley & Krieg, 1984). To speciate an organism means to give that organism a name which in turn means that the organism is assigned to a pre-existing taxonomic group. Woese *et al.* (1984) has argued that the use of names (of Latin and Greek derivation) leads to the rigidification of the existing system of classification that now impedes progress in microbiology. The existing classification of micro-organisms is based, in the main, on phenotypic characteristics. Thus, the name given to an organism would be indicative of what the organism did (expressed phenotype) and would suggest a phenotypic relationship between all organisms of the same name. In the case of *Pseudomonas*, a group that is now classified according to its genotypic structure, this may not be true (Stanier *et al.*, 1966; Woese *et al.*, 1984). According to Stackebrandt & Woese (1984), systems of bacterial classification based on phenotypic characters "*will be judged historically as counter-productive and distorted conceptual structures*".

In the first instance within this study classical identification methods (physical, biochemical and morphological) were applied to food and environmental *Pseudomonas* isolates with a view to speciating them. However, little success was seen with a significant proportion of the environmental isolates. The API 20NE system was used to aid the speciation of unknown *Pseudomonas* isolates from various environments as the tests were standardised and simple to use. Most of the unknowns were accurately described to the

genus level with approximately 19% not speciated and 5% were known to be incorrectly identified. In view of the present structure of the genus perhaps the expectation of speciation of all of the food and environmental isolates with a nutritional assay of 21 tests, is now, in hindsight, over-ambitious, although the manufacturer's would have you believe otherwise. However, the API 20NE system was reliable and faster than performing numerous classical tests. Costas *et al.* (1992) evaluated the API 20NE system with 146 strains representing 15 medically important *Pseudomonas* species. They found that 90.4% of the test strains were correctly identified, 5.5% not identified and 4.1% incorrectly identified. *Pseudomonas* isolates obtained from hospital patients tend to be more easily identifiable by the API 20NE system than environmental isolates possibly because the hospital isolates usually fall into well-characterised groups. Isolates from diverse environments are more likely to fall outside the characterised groups and/or produce atypical results due to environment-driven phenotypic adaptations.

Numerical taxonomic analysis of a large number of carbon source oxidation data allowed the grouping of the *Pseudomonas* isolates which was strongly influenced by the type of environment from which they were isolated. The commercial availability of standardised nutritional tests (e.g. Biotype-100, BioMerieux) in easy formats makes the conduct of large scale analysis easier, more rapid, reliable and less time-consuming. However, the inability of the Biolog MicroPlate™ data to group some of the genomic species into a single taxon or cluster (e.g. *Ps.fluorescens*, which was distributed within six out of ten taxa) indicates that additional taxonomically relevant tests were needed. The inclusion of the API 20NE data, which were non-ovelapping tests, in the numerical analysis may have further aided identification.

In practice, phenotypic characterisation of *Pseudomonas* has become ineffectual unless very large numbers of tests are conducted. However, the report of the Ad Hoc Committee on the Reconciliation of Approaches to Bacterial Systematics (Wayne *et al.*, 1987) stressed that any phylogenetically based taxonomic scheme must also show phenotypic

consistency. Comparative physiology and biochemistry of new and known strains will always be necessary since *Pseudomonas* are often more important for what they do than for what they are.

The genotype and phenotype are obviously related and in practice, related phenotypic properties generally point to a group of closely related species (Brosch *et al.*, 1996). At this point DNA:DNA hybridisation, nucleic acid probe hybridisation, or amplification of a species-specific DNA target are applied as the definitive method for identification. When the initial identification is too vague (e.g. pseudomonad) the molecular techniques become labour intensive as many probes, amplification primers or reference DNA samples are required (Brosch *et al.*, 1996).

Future identification of unknown *Pseudomonas* isolates, within the current phenotypic classification scheme (Palleroni, 1984), could be simplified by initially assigning the isolate to one of the genotypic groups. Assigning an isolate to a specific rRNA homology group would reduce the number of species with which it is to be compared. However, the arrangement of the *Pseudomonas* genus is still in flux. Some *Pseudomonas* found in particular environments e.g., meat were not represented in the initial studies of Stanier *et al.* (1966) from whose work the current classification evolved. In time, with the inclusion of more strains isolated from different types of environments, the classification of the genus will become more robust. At this point chemotaxonomic markers such as fatty acids and quinones could be used as good phenotypic indicators of genotype (Oyaizu & Komagata, 1983).

The definitive answer to the identification of unknown isolates that belong to a group that is classified according to genotype must be given by a molecular procedure. Numerous studies into the relatedness of micro-organisms have used ribosomal RNA or DNA as the basis for their investigations and the ribosomal genes have now become the gold standard for such analyses. Other macromolecules have been investigated for their

usefulness to determine phylogenetic relationships. Amongst these is the beta sub-unit of ATP-ase (Ludwig *et al.*, 1993), chaperonin (Viale *et al.*, 1994) and RNA polymerase (Zillig *et al.*, 1989). These alternatives to 16S rRNA should be universally distributed amongst bacteria, their genes should not be transmitted horizontally, and their molecular evolution rate should be comparable or higher than that of 16S rRNA (Vandamme *et al.*, 1996) hence they fulfil the necessary criteria for a universal 'molecular clock'. When investigated ribotyping proved highly discriminating when used to identify wild type *Pseudomonas* species. The discrimination seen was to the level of sub-species as the technique produced unique patterns for strains of the same species. In order to elucidate relationships between isolates the banding patterns produced would have to be further analysed using numerical taxonomic methods or compared to those of a larger database. Such databases are in use e.g. associated with the Riboprinter (DuPont) but to date they have mainly been located at specialist centres with strain characterisers as a commercial service.

The developed ARDRA protocol was sufficiently discriminating to distinguish between species and the protocol would have been applied more widely within this study if time had permitted. A definitive answer to *Pseudomonas* identification could have been achieved by sequencing the amplimers (1kbp) obtained with the 16S rDNA primers as demonstrated by Moore *et al.* (1996) who used a 1.5kbp amplimer. However, the cost of such analyses, that was not routinely conducted in-house, was prohibitive (approximately £5-£6 per sample). However, as an alternative to using API and Biolog systems it would be cost effective if this was your starting point.

The advancement of molecular techniques means that whole genome sequences can be determined and the phylogenetic relationships between organisms can be elucidated precisely. However, classifying organisms by chromosome analysis leads to problems when microbiologists try to phenotypically differentiate between organisms belonging to different phylogenetically defined groups. Organisms with the same phenotype, within a particular environment, could have evolved through distinct phylogenetic routes.

Accordingly, phenotypically identical organisms could be classified differently. The cross-reactions of the antisera used in this study were found to relate with the source of the *Pseudomonas* isolates rather than the species. Numerical taxonomic studies of pseudomonads based on nutritional data objectively grouped the isolates from several environmental sources with the resulting groups formed strongly influenced by the origin of the isolates rather than species. Thus phenotypic characterisation did not reflect phylogeny. This current situation with the *Pseudomonas* genus reflects the fact that classification is achieved genotypically whilst routine identification is attained phenotypically, at times, as found in this study, with little success.

The surveillance of natural populations within complex environments was successfully mediated by antibody-linked probes. The antiserum used was raised against an isolate that originated from raw milk, which was a related environment to that against which the antiserum was applied. Antibody-linked probes can be applied to other complex bacterial systems such as biofilms. Immobilised consortia behave differently to free living organisms. Advantages of this lifestyle are the higher availability of nutrients on surfaces and the possibility of optimal long-term positioning in relation to other micro-organisms or physicochemical gradients. Specific analyses of spatial distribution in such systems can only be resolved with *in situ* techniques like immuno-fluorescence or *in situ* hybridisation.

Antisera raised against bacteria recognise and bind to antigenic phenotypic structures (e.g. LPS). The phenotypic structures that they bind to may not be taxonomically relevant. Hence, the antibodies raised in this study could not be used for identification purposes. The antibodies raised could only be used to follow a sub-set of a population originating from the same environment as the immunising strain or used within model systems with known microflora.

A major weakness with applying antibody-linked probes to follow the growth of natural populations was that the dot-blot assays could produce subjective data. The test

organisms produced a spectrum of colour intensities and negative results were at times difficult to deduce by eye. The employment of computer equipment and software to analyse the colour intensities of the spots on the immuno-blots could lead to the introduction of cut-off values. Cut-off values would clearly define positive and negative reactions. Such criteria are already an integral part of other immuno-chemical assays such as ELISA. The combination of standardised methods and objective interpretation would yield reliable qualitative and quantitative data. The variation seen within the data would then solely be due to the distribution of epitopes on the bacterial surface cross-reacting with the antibody.

The mechanism of cross-reactivity of the antisera raised against multiple immunogens in the competitive ELISA was unclear. The competitive ELISA exhibited selective specificity in the presence of Edward (*Ps. fluorescens*) antiserum when one of the *Ps. fluorescens* isolates used to raise the antiserum was bound to the microtitration plate. A positive reaction was seen only when the bound isolate and the isolate in suspension were the same. If the isolate in suspension was different to that bound then a negative reaction was seen i.e. all the antibodies bind to the plate, although both isolates were used to raise the antiserum. All theories postulated to explain the phenomenon pertaining to the antibody population proved unsound. The answer may not be immuno-chemical but may be biological and relate to the nature of the particular organisms used. The antiserum raised against food spoilage *Pseudomonas* was found to bind onto the bacterial cell surface via the lipopolysaccharide and extracellular polysaccharide. According to Rivera *et al.* (1988), who studied the LPS structure of *Ps. aeruginosa*, *Pseudomonas* species are known to produce more than one LPS-like molecule with differing antigenic properties. When individual isolates bind to the microtitration plate surface, different LPS-antibody-binding sites or epitopes may become available that are more antigenic than the binding sites of the same organism in suspension. The population of antibodies that would otherwise displace the cells in suspension preferentially bind to the bound cells. The organism in suspension may then try to attach to the organisms bound to the surface to create a

biofilm (the reaction is incubated for 30 min). When the organism is beginning to attach to a similar isolate then the attaching isolate also presents highly antigenic LPS or epitopes to the antibody which on washing displaces the attaching organism in suspension. If the organism in suspension differs to that bound to the plate then the LPS or epitopes that it presents to a non-self bound organism during attachment may be different and less antigenic and therefore the antibody population binds preferentially to the bound organism. The hypothesis outlined can account for the selective specificity phenomenon but is purely speculative. The consequences of selective specificity are that a single polyclonal population could be manipulated to specifically detect several bacterial isolates greatly increasing the utility of the antiserum.

The developed near-food-grade model emulsion allowed the study of *Pseudomonas* growth *in situ* under standardised conditions. The effect of the structure of the 70% oil-in-water emulsion on the growth of *Pseudomonas*, which led to the formation colonies was demonstrated. The data generated supported the work reported by Gunning (1996). The optimisation of multiple antibodies to detect two or more different organisms within the near-food-grade model emulsion was not successful. Had multiple staining protocols been attained then investigations into the bacterial composition of the colonies could have been initiated. The possibility of closely associated bacteria of different species forming a single colony (multixenic colony) within food matrices may have been fully explored. The implications of the formation of multixenic colonies within food may have food safety consequences if pathogens and harmless spoilage organisms could form a single colony. Within a multixenic colony, a pathogen may avoid detection when the food is subsequently assessed microbiologically.

The *in situ* antibody-linked staining techniques that were developed with the near-food-grade model emulsion could be applied to real food systems. The behaviour of *Pseudomonas* species within the natural food environment, could be monitored such that ecologically relevant traits could be identified within a complex heterogeneous population

and matrix. In this manner, naturally occurring microflora could be located within natural communities, which would negate the need to mimic the natural food environment and would overcome the problem of non-culturable environmental isolates. Additionally, following natural populations *in situ* would overcome problems associated with introduced organisms. As found in this study introduced organisms may not behave in a similar way to natural community members until a period of adaptation has occurred.

The development of sensitive non-destructive methods for monitoring bacteria in laboratory model systems and natural environments is universally important. Manipulated bacteria containing green fluorescent protein (GFP) have been used to monitor the attachment of *Ps. fluorescens* to the roots of tomato seedlings *in situ* (Bloemberg *et al.*, 1997). The expression of GFP within a mixed microbial population was monitored using fluorescence microscopy. The fluorescent cells remained visible on the root tip for up to 9 days after inoculation and were first detected 2 hours after inoculation of the germinated seedling. Even though the authors chose a *Ps. fluorescens* strain that was known to colonise tomato seedling roots to initially manipulate the strain used was an introduced organism. As stated previously, introduced organisms may not behave in a manner comparable to members of the natural microflora.

New microscopy techniques such as scanning confocal laser microscopy (that can optically section complex bacterial populations; Lawrence *et al.*, 1991) and atomic force microscopy (which can visualise living cells and macromolecular interactions; Kirby & Morris, 1994) are also potentially useful tools that microbiologists, environmental scientists and ecologists can utilise for studying complex biological systems.

The aim of this project was to develop antibody-linked probes that could follow a known population of psychrotrophic *Pseudomonas* species within a complex food system. This aim was successfully achieved. The characterisation of the sub-set of *Pseudomonas* species that cross-reacted with the antibodies was an objective that was attained. However, the

characterisation techniques did not yield definitive identifications in all cases although schemes for nutritional relatedness of the micro-organisms were established.

The application of the antibody-linked probes to real and model food systems highlighted the reliability and versatility of the antibodies. However, this work has demonstrated that in a phenotypically complex group like the pseudomonads antibodies (polyclonal or monoclonal) must be screened, characterised and optimised so that they can be applied appropriately to answer specific experimental questions.

RAW BIOLOG DATA
(including duplicates)

SAMPLE NUMBER	I- ERYTHRITOL	D- MELIBIOSE	ACETIC ACID	P- HYDROXYPHENYL ACETIC ACID	BROMO SUCCINIC ACID	L- HISTIDINE	UROCANIC ACID	A- CYCLODEXTRIN	D- FRUCTOSE	B- METHYL-D-GLUCOSIDE	CIS- ACONITIC ACID	ITACONIC ACID	SUCCINAMIC ACID	HYDROXY-L-PROLINE	INOSINE	DEXTRIN	L- FUCOSE	D- PSICOSE	CITRIC ACID	A- KETO-BUTYRIC ACID	GLUCURONAMIDE	L- LEUCINE	URIDINE	GLYCOGEN	D- GALACTOSE	D- RAFFINOSE	FORMIC ACID	A- KETO-GLUTARIC ACID	ALANINAMIDE	L- ORNITHINE	THYMIDINE	TWEEN 40	GENTIOBIOSE	L- RHAMNOSE	D- GALACTONIC ACID LACTONE	A- KETO-VALERIC ACID	D- ALANINE	L- PHENYLALANINE	PHENYLETHYLAMINE	TWEEN 80	A-D- GLUCOSE	D- SORBITOL	D- GALACTURONIC ACID	D,L- LACTIC ACID	L- ALANINE	L- PROLINE	PUTRESCINE
1	0	0	1	1	1	1	1	0	1	0	1	1	1	1	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	1	0	0	0	1	0	1	1	1	1	0	1	0
1.1	0	0	0	0	1	1	1	0	1	0	1	1	0	1	1	0	0	0	1	1	0	0	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	1	1	1	0
2	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	0	0	1	1	1	1	1	1	0	1	0	1	1	1	1	0	1	0	0	1	1	1	1	1	1	1	0
2.1	0	0	0	1	1	1	1	0	1	0	1	1	1	1	1	0	0	1	1	1	1	1	1	0	1	0	1	1	1	1	0	1	0	0	1	1	1	1	1	1	1	1
3	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	0	0	0	1	0	1	1	1	0	1	0	1	1	1	1	0	1	0	0	1	1	1	1	1	1	1	0
3.1	1	0	0	1	1	1	1	0	1	0	1	1	0	1	1	0	0	0	1	1	0	1	1	0	1	0	1	1	1	1	0	1	0	0	1	0	1	0	0	1	1	1	1	1	1	0
4	0	0	1	1	1	1	1	0	1	0	1	0	1	1	1	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	0	1	0	0	1	1	1	0	0	1	1	1	1	1	1	0
4.1	0	0	0	1	1	1	1	0	1	0	1	0	1	1	1	0	0	1	1	0	0	1	1	0	1	0	1	0	0	1	1	1	0	1	0	0	1	0	1	0	0	1	1	1	1	0
5	1	0	1	1	1	1	1	0	1	0	1	1	1	1	1	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	0	1	0	0	1	1	1	0	0	1	1	1	1	1	1	0
6	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	0	0	1	1	1	1	1	0	0	1	0	1	1	1	1	0	1	0	0	1	0	1	0	0	1	1	1	1	1	1	0
6.1	1	0	0	1	1	1	1	0	1	0	1	1	1	1	1	0	0	1	1	1	1	1	1	0	1	0	0	1	1	1	0	1	0	0	1	0	1	0	0	1	1	1	1	1	1	0
7	0	0	1	1	1	1	1	0	0	0	1	1	1	1	1	0	0	0	1	0	1	0	1	0	1	0	0	1	0	0	0	1	0	0	1	0	1	0	0	1	1	1	1	1	1	0
7.1	0	0	0	1	1	1	1	0	0	0	1	1	0	1	1	0	0	0	1	0	0	0	0	0	1	0	1	1	0	1	0	1	0	0	1	0	0	0	0	1	1	1	1	1	1	0
8	1	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	0	1	1	1	1	0	0	1	1	1	1	1	0
8.1	1	0	0	1	1	1	0	0	1	0	1	1	0	1	1	0	0	1	1	0	1	1	1	0	1	0	1	1	1	1	0	1	0	0	1	1	1	0	0	1	1	1	1	1	1	0
9	1	1	1	1	1	1	1	0	1	0	1	0	1	1	1	0	0	1	1	1	1	1	1	0	1	0	1	1	1	0	0	1	0	0	1	1	1	0	0	1	1	1	1	1	1	0
9.1	1	1	1	1	1	1	1	0	1	0	1	0	1	1	1	0	0	1	1	0	1	1	1	0	1	0	0	1	0	0	0	1	0	0	1	0	1	0	0	1	1	1	1	1	1	0
10	0	0	0	0	1	1	1	1	0	1	0	1	1	0	1	1	0	0	0	1	0	0	0	1	0	1	0	0	1	0	1	0	1	0	0	1	0	1	0	0	1	1	1	1	1	0
11	0	0	1	1	0	1	1	1	0	0	1	0	1	0	1	0	1	0	0	1	1	1	0	0	1	0	0	0	0	0	1	1	1	0	0	1	0	0	0	0	1	1	1	1	1	1
11.1	0	1	1	1	0	1	1	1	0	0	1	0	1	0	1	0	1	0	0	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0	1	0	1	1	0	1	1	1	1	1	1	1
12	1	0	1	1	1	1	1	1	0	1	0	1	1	0	1	1	0	0	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	0	1	0	1	0	0	1	1	1	1	1	1	0
12.1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	0	1	1	0	1	1	1	0	1	1	1	1	1	1	1	0	1	0	1	0	1	1	1	1	0	0	1	1	0
13	0	0	1	0	1	1	1	1	0	1	0	1	1	1	1	1	0	1	1	1	1	0	1	1	1	1	0	1	0	1	1	0	1	0	1	0	1	0	0	0	0	1	0	0	1	1
13.1	0	0	0	0	0	1	1	1	0	1	0	1	1	1	1	1	0	1	1	1	1	1	1	0	0	1	0	1	1	1	1	1	0	1	0	0	0	0	1	1	0	0	0	1	1	1
14	0	1	1	0	1	1	0	0	1	0	1	1	1	1	1	0	1	1	1	0	1	0	0	0	1	0	0	1	0	1	0	1	0	0	0	0	1	1	0	0	0	1	0	0	1	1
14.1	0	0	0	0	1	1	0	0	1	0	1	1	1	1	0	0	1	1	1	0	0	1	0	0	1	0	1	1	0	0	0	1	0	1	0	0	0	1	1	0	0	0	1	0	0	1
15	1	0	1	1	1	1	1	0	1	0	1	0	1	1	1	0	0	0	1	1	0	1	1	0	0	0	1	1	0	1	0	1	0	0	0	0	0	0	0	1	1	0	0	1	1	0
15.1	0	0	0	0	1	1	1	1	0	0	0	1	0	1	1	1	0	0	0	1	1	0	0	1	0	0	0	0	0	0	1	0	1	0	1	0	0	0	1	1	0	0	1	1	1	0
16	0	0	1	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
17	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0
17.1	0	0	0	0	1	1	1	1	0	1	0	1	1	1	1	1	0	0	0	1	0	0	1	0	0	0	0	1	1	0	1	0	1	0	0	0	0	0	1	0	0	1	1	0	1	1
19	0	0	1	0	1	1	1	1	0	0	0	0	0	0	1	1	0	1	1	0	0	0	0	0	0	1	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	1	0	0	1	1	1	0
19.1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	1	0
20	0	0	1	0	1	1	1	1	0	1	0	1	0	1	1	0	0	1	1	1	0	0	0	0	0	1	0	1	1	0	0	0	1	0	0	0	0	1	0	0	1	1	0	0	1	1	1	0
21	0	0	1	0	1	1	1	1	0	1	0	1	0	1	1	0	0	1	1	1	1	0	0	0	0	1	0	1	1	1	1	0	1	0	0	1	1	1	0	0	1	1	0	1	1	1	1	1
22	0	0	0	0	0	1	1	1	0	1	0	1	0	1	1	1	0	1	1	1	1	0	1	0	1	0	1	0	1	0	0	1	0	1	0	1	0	0	1	0	0	0	0	0	1	0	1	1	1	1
23	1	0	0	0	1	1	1	1	0	1	0	1	0	1	1	1	0	1	1	1	1	0	1	1	0	1	0	1	0	1	1	0	1	0	1	0	1	0	0	1	0	1	0	0	0	1	0	0	1	1	1	1
24	0	1	1	0	1	1	1	1	0	1	0	1	0	1	1	1	0	1	1	1	1	1	1	1	1	0	1	0	1	1	0	1	0	1	0	1	0	0	1	0	1	0	0	1	1	0	0	1	1	1	1	1
25	0	0	1	0	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	0	1	0	1	1	1	1	1	0	1	0	1	0	0	1	1	1	0	0	0	1	1	1	1	1	1	1	1
26	0	0	1	0	1	1	1	1	0	1	0	1	0	1	1	1	0	1	1	1	0	0	0	0	0	1	0	0	1	0	1	0	1	0	1	0	0	1	0	0	0	0	0	0	1	0	1	1	1	1	1	0

RAW BIOLOG DATA
(including duplicates)

APPENDIX A

SAMPLE NUMBER	N- ACETYL GALACTOSAMINE	M- INOSITOL	SUCROSE	D- GLUCONIC ACID	MALONIC ACID	L- ALANYL-GLYCINE	L- PYROGLUTAMIC ACID	2- AMINO ETHANOL	N- ACETYL-D-GLUCOSAMINE	A-D- LACTOSE	D- TREHALOSE	D- GLUCOSAMINIC ACID	PROPIONIC ACID	L- ASPARAGINE	D- SERINE	2,3- BUTANEDIOL	ADONITOL	LACTULOSE	TURANOSE	D- GLUCURONIC ACID	QUINIC ACID	L- ASPARTIC ACID	L- SERINE	GLYCEROL	L- ARABINOSE	MALTOSE	XYLITOL	A- HYDROXYBUTYRIC ACID	D- SACCHARIC ACID	L- GLUTAMIC ACID	L- THREONINE	D,L-A- GLYCEROL PHOSPHATE	D- ARABITOL	D- MANNITOL	METHYL PYRUVATE	B- HYDROXYBUTYRIC ACID	SEBACIC ACID	GLYCYL-L-ASPARTIC ACID	CARNITINE	GLUCOSE-1-PHOSPHATE	D- CELLOBIOSE	D- MANNOSE	METHYL SUCCINATE	G- HYDROXYBUTYRIC ACID	SUCCINIC ACID	GLYCYL-L-GLUTAMIC ACID	G- AMINO BUTYRIC ACID	GLUCOSE-6-PHOSPHATE
1	0	1	0	1	0	0	1	1	0	0	0	0	1	1	0	0	0	1	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1	1	1	1	1	0	0	0	0	0	1	1	0	1	0
1.1	0	1	0	1	0	1	1	1	0	0	0	1	1	1	1	0	0	1	0	0	1	1	1	1	1	0	0	0	1	1	1	0	1	1	1	1	1	0	0	0	0	0	1	1	0	1	0
2	0	1	1	1	1	1	1	1	0	0	0	1	1	1	1	0	1	0	0	0	1	1	1	1	1	0	0	0	1	1	1	0	1	1	0	1	0	0	0	0	0	0	1	1	0	1	0
2.1	0	1	0	1	1	1	1	1	0	0	0	1	1	1	1	0	1	0	0	1	1	1	1	1	1	0	1	1	1	1	1	0	1	1	1	1	0	1	0	0	0	1	1	0	0	1	0
3	0	1	0	1	1	1	1	0	1	1	0	0	1	1	1	0	0	1	0	0	1	0	1	1	1	1	0	0	1	1	1	1	0	1	1	1	0	1	0	0	1	1	0	0	1	1	0
3.1	0	1	0	1	1	1	1	0	1	1	0	1	1	1	1	0	0	1	0	1	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	1	1	0	1	1	0	0	1	1	0	1	0
4	0	0	0	1	0	1	1	1	1	0	1	1	1	1	1	0	0	1	0	0	1	1	1	1	1	0	0	0	1	1	1	0	1	1	0	1	0	0	0	0	0	0	1	1	0	0	0
4.1	0	0	0	1	1	1	1	1	1	0	1	1	1	1	1	0	1	0	0	1	1	1	1	1	1	0	1	0	1	1	1	1	0	1	1	1	1	1	1	0	0	1	1	0	1	1	0
5	0	1	1	1	1	1	0	1	1	0	1	1	1	1	1	0	1	0	0	1	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	0	1	0	1	1	0	0	1	1	0	1	0
6	0	1	1	1	1	1	1	1	1	0	0	1	1	1	1	0	0	0	0	0	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	0	1	0	0	0	0	0	1	1	0	1	0
6.1	0	1	0	1	1	1	1	1	1	0	1	1	1	1	1	0	0	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	0	1	0	1	1	0	0	1	1	0	1	0
7	0	1	0	1	1	1	1	1	0	0	0	0	1	0	1	0	0	1	0	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1	1	0	1	0	0	0	0	0	1	1	0	1	0
7.1	0	1	0	1	1	0	1	1	0	0	1	1	1	1	1	0	0	1	0	1	1	1	1	1	1	1	0	0	0	1	1	0	0	1	1	1	1	0	0	1	0	0	1	1	0	1	0
8	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	0	0	0	1	1	0	1	1	0	0	1	1	0	1	0
8.1	0	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	0	0	1	1	1	1	1	1	0	0	1	1	1	1	0	0	0	1	1	0	1	1	0	0	1	1	0	1	0
9	0	1	0	1	1	1	1	1	1	1	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0	0	1	1	1	1	0	0	1	0	0	1	1	0	0	1	1	0	1	0
9.1	0	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	0	0	1	0	1	1	1	1	1	1	1	0	0	0	1	1	1	0	1	1	1	1	0	0	1	0	0	1	1	0	1	0
10	0	1	0	1	1	1	1	1	1	0	0	0	0	1	0	1	0	0	1	0	0	1	1	1	1	1	1	0	0	0	0	1	1	1	0	0	1	1	1	1	0	0	0	0	1	1	0
11	0	1	0	1	0	1	0	1	1	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0
11.1	0	1	0	1	0	1	0	1	1	1	0	0	1	0	1	1	1	1	0	0	0	1	0	0	1	1	1	1	1	0	1	1	0	1	1	0	0	0	0	1	1	1	0	0	1	1	1	0
12	0	1	0	1	1	1	1	1	1	0	0	0	1	1	1	1	0	0	1	0	0	1	1	1	1	1	1	0	0	0	1	1	0	0	1	1	1	1	0	0	0	0	1	1	0	1	1	0
12.1	0	1	0	1	1	1	1	1	1	0	0	1	1	1	1	1	0	0	1	0	1	1	1	1	1	1	1	0	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	1	1	0	1	0
13	0	0	1	1	0	1	1	0	0	0	1	0	1	1	1	1	0	0	0	0	0	1	1	0	1	1	1	1	1	0	0	1	1	1	0	0	0	1	1	0	0	0	0	0	1	1	1	0
13.1	0	0	1	1	0	1	1	0	0	0	1	0	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	0	0	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	1	1	1	0
14	0	0	1	1	0	1	1	0	0	0	1	0	1	1	1	1	0	0	0	0	0	1	0	1	1	1	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0
14.1	0	1	1	1	0	1	1	0	0	0	1	0	1	1	1	1	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	1	1	1	0	0	0	1	0	0	0	0	0	0	1	1	0	0
15	0	1	0	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	1	0	0	0	1	1	1	1	0	0	0	0	0	1	0	1	1	1	1	1	1	0	0	1	0	0	1	1	1	0
15.1	0	1	0	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	1	0	0	0	1	1	1	1	0	0	1	1	0	1	1	0	1	1	1	1	1	0	0	1	0	0	0	1	1	0
16	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0
17	0	0	0	1	1	0	1	1	1	0	0	0	1	1	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	1	0	1	0	1	0
17.1	0	0	0	1	1	1	1	1	1	0	0	0	1	1	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	1	0	0	0	1	0	1	0
19	0	1	0	1	0	1	1	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	1	1	1	1	1	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	1	0
19.1	0	1	0	1	0	0	1	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0
20	0	1	0	1	0	1	1	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	0
21	0	1	0	1	0	1	1	1	0	0	0	0	0	1	1	1	0	0	0	0	0	1	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	1	1	0	0	1	0	0	1	1	1	0	0
22	0	1	0	1	0	0	1	0	0	0	0	0	0	1	1	1	0	0	0	0	0	1	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	1	1	0	0	1	0	0	1	1	0	1	0
23	0	1	0	1	0	1	1	1	0	0	0	0	0	1	1	1	0	0	0	0	0	1	1	1	1	1	1	0	0	0	1	1	0	0	0	0	0	1	0	0	0	1	0	0	1	1	0	1	0
24	0	1	0	1	0	1	1	1	1	0	0	0	0	1	1	1	0	0	0	1	1	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	1	1	0	0	0	1	0	0	1	1	0	1	0
25	0	1	0	1	0	1	1	1	0	0	0	0	0	1	1	1	0	0	0	0	1	1	1	1	1	1	1	0	0	1	1	1	0	1	0	0	1	1	0	1	1	0	0	1	1	0	1	1	0
26	0	1	0	1	0	1	1	0	0	0	0	0	0	1	1	1	0	0	0	0	0	1	0	1	0	1	1	0	0	0	0	1	1	0	0	0	0	0	1	1	0	0	0	0	1	1	0	1	0

RAW BIOLOG DATA

(including duplicates)

SAMPLE NUMBER	I- ERYTHRITOL	D- MELIBIOSE	ACETIC ACID	P- HYDROXYPHENYL ACETIC ACID	BROMO SUCCINIC ACID	L- HISTIDINE	UROCANIC ACID	A- CYCLODEXTRIN	D- FRUCTOSE	B- METHYL-D-GLUCOSIDE	CIS- ACONITIC ACID	ITACONIC ACID	SUCCINAMIC ACID	HYDROXY-L-PROLINE	INOSINE	DEXTRIN	L- FUCOSE	D- PSICOSE	CITRIC ACID	A- KETO-BUTYRIC ACID	GLUCURONAMIDE	L- LEUCINE	URIDINE	GLYCOGEN	D- GALACTOSE	D- RAFFINOSE	FORMIC ACID	A- KETO-GLUTARIC ACID	ALANINAMIDE	L- ORNITHINE	THYMIDINE	TWEEN 40	GENTIOBIOSE	L- RHAMNOSE	D- GALACTONIC ACID LACTONE	A- KETO-VALERIC ACID	D- ALANINE	L- PHENYLALANINE	PHENYLETHYLAMINE	TWEEN 80	A-D- GLUCOSE	D- SORBITOL	D- GALACTURONIC ACID	D,L- LACTIC ACID	L- ALANINE	L- PROLINE	PUTRESCINE
27	0	0	1	0	1	1	1	0	1	0	1	0	1	1	1	1	1	1	1	0	0	0	0	0	1	0	1	1	0	0	1	0	0	0	1	1	1	0	0	1	1	1	1	0
28	1	0	1	1	1	1	1	0	1	0	1	1	0	1	1	0	0	1	1	0	1	1	1	0	1	0	1	1	0	1	0	1	0	0	1	1	1	1	1	1	1	1	1	0
29	1	0	1	1	1	1	1	0	1	0	1	1	1	1	1	0	1	1	1	0	1	1	1	0	1	0	1	1	0	1	0	1	0	0	1	0	1	0	1	1	1	1	1	0
30	1	0	1	1	1	1	1	0	1	0	1	1	0	1	1	0	0	1	1	0	1	0	1	0	1	0	1	1	0	1	0	1	0	0	1	0	1	0	0	1	1	1	1	0
31	0	0	0	0	0	1	1	1	1	0	1	0	1	0	0	1	0	0	1	1	1	0	0	0	0	0	1	0	0	1	0	1	0	1	0	0	0	0	1	0	0	1	1	0
32	1	0	1	1	1	1	1	1	1	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0	1	0	1	1	0	1	0	1	0	0	1	0	1	0	0	1	1	1	1	0
33	0	0	0	0	1	0	0	1	1	0	0	0	1	0	1	1	1	0	0	0	1	0	0	0	0	0	1	0	0	1	0	1	0	1	0	0	0	0	0	0	1	1	0	1	0
34	0	1	1	1	1	1	0	1	0	1	1	1	1	0	1	1	1	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	0	1	0	0	1	0	1	1	1	0	1	1	1
36	0	0	0	1	1	1	0	1	0	1	0	1	0	1	1	1	1	0	0	1	1	0	1	1	1	0	1	0	1	1	0	1	0	1	0	0	1	0	1	0	0	1	1	1	0
38	0	0	0	0	1	1	0	1	0	0	0	0	1	0	1	1	1	1	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0	0	1	0	1	1	0	1	0
38.1	0	0	0	0	0	1	0	1	0	0	0	0	1	0	1	1	1	1	0	0	0	1	1	0	1	1	0	1	0	0	1	1	1	0	1	0	0	1	0	1	0	1	1	1	0
39	0	0	1	1	1	1	0	1	0	0	0	0	1	0	1	1	1	1	0	0	0	1	0	1	1	0	0	1	0	0	1	1	1	0	1	0	0	1	0	1	0	1	1	1	0
41	0	0	0	0	1	1	1	1	0	0	0	0	1	0	1	1	1	1	0	0	0	1	0	0	1	0	0	0	0	0	0	1	0	1	0	1	0	0	0	1	0	0	1	1	0
42	1	0	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	0	1	1	1	0	1	1	0	1	1	0	1	0	1	0	1	0	1	0	0	1	0	1	1	1	1	1
43	0	0	1	0	1	1	1	1	0	1	0	1	0	1	0	1	0	1	1	1	1	0	1	0	0	0	0	0	1	1	0	1	0	1	0	0	0	0	1	1	1	0	1	1	0	1	1
44	0	0	1	0	1	1	1	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	1	1
45	0	0	1	0	1	1	0	0	1	0	1	0	1	0	0	0	0	0	1	1	0	1	1	1	1	0	1	0	1	1	1	1	0	0	1	0	0	0	0	0	0	1	1	0	0	1	1
46.1	0	0	1	0	1	1	1	0	1	0	1	0	0	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	1	1
47	0	0	1	1	1	0	1	0	1	0	1	0	1	1	1	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0	1	0	0	1	0	1	1	1	1	1	1	1	1	0
48	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
50	0	1	0	0	1	1	1	0	1	0	1	1	1	1	1	0	0	1	1	1	1	1	1	0	1	0	0	1	0	0	0	0	0	0	1	1	0	1	0	0	0	1	1	1	1	0	0
51	0	0	1	1	1	1	1	1	0	1	0	1	0	1	1	1	1	0	0	1	1	1	1	1	1	0	1	0	1	1	0	1	0	1	0	1	1	1	1	0	0	1	1	1	1	1	0
52	0	0	0	0	1	1	1	0	0	0	0	0	1	1	1	1	1	0	0	0	1	0	0	1	1	0	0	1	0	0	0	1	1	1	0	1	0	1	0	0	1	1	0	0	1	1	0
53	0	0	1	1	1	1	1	0	0	1	0	1	0	1	1	1	1	0	0	0	1	1	1	1	1	1	0	1	0	0	0	1	1	0	0	1	0	0	1	1	0	0	1	1	1	1	1
53.1	0	0	1	1	1	1	0	0	0	1	0	1	0	1	1	1	1	0	0	1	1	0	1	1	1	1	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	1	1	0	0	1	0
54	0	0	1	1	1	1	1	1	0	1	0	1	0	1	1	1	1	0	0	1	1	1	1	1	1	1	0	1	0	1	1	1	0	0	1	0	1	0	1	0	0	1	1	1	1	1	0
55	0	0	1	1	1	0	1	0	1	0	1	0	1	1	1	0	0	1	1	1	1	1	1	1	0	1	0	0	1	0	0	0	1	0	0	1	0	1	0	0	1	1	1	1	1	1	1
56	0	0	1	1	1	1	1	1	0	1	0	1	0	1	1	1	0	0	0	1	0	0	1	1	0	1	0	0	1	0	1	0	1	0	1	0	1	0	1	0	1	0	1	1	1	1	0
57	0	0	1	0	1	1	1	1	0	1	0	1	0	1	1	1	0	0	1	1	0	0	1	1	0	1	0	1	0	1	1	1	1	0	1	0	0	1	1	1	0	0	1	1	1	1	1
58	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1	1	0	0	1	1	0	0	0	1	0	0	1	0
59	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	0	0	1	1	1	1	1	1	1	1	1	1	1
61	1	1	1	1	0	1	1	1	1	1	0	0	1	1	1	1	0	1	1	0	0	0	1	1	1	1	1	1	0	1	0	1	1	1	1	0	0	1	1	0	1	1	1	1	1	1	0
65	0	0	1	1	1	1	1	0	1	0	1	0	1	1	1	0	0	1	1	0	0	1	1	0	1	0	1	1	1	1	0	1	0	0	1	0	1	0	0	1	1	0	0	1	1	1	0
66	1	0	1	1	1	1	1	0	1	0	1	1	1	1	1	0	0	1	1	1	0	1	1	0	1	0	1	1	1	1	1	0	1	0	1	1	0	1	0	0	1	1	0	0	1	1	0
67	0	0	0	0	1	1	0	0	0	0	0	0	1	1	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	1	0	1	1	0	0	0	1	1	0	0	1	1	0
68	0	0	1	0	1	0	1	0	1	0	1	0	0	1	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	0	1	0	0	0	1	0	1	0	0	0	0	1	1	0	0	1	0
69	0	0	0	1	1	1	1	0	1	0	1	1	1	1	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1	1	1	0	1	0	1	1	0	1	0	0	1	1	0	0	1	0

SAMPLE NUMBER	N- ACETYL GALACTOSAMINE	M- INOSITOL	SUCROSE	D- GLUCONIC ACID	MALONIC ACID	L- ALANYL-GLYCINE	L- PYROGLUTAMIC ACID	2- AMINO ETHANOL	N- ACETYL-D-GLUCOSAMINE	A-D- LACTOSE	D- TREHALOSE	D- GLUCOSAMINIC ACID	PROPIONIC ACID	L- ASPARAGINE	D- SERINE	2,3- BUTANEDIOL	ADONITOL	LACTULOSE	TURANOSE	D- GLUCURONIC ACID	QUINIC ACID	L- ASPARTIC ACID	L- SERINE	GLYCEROL	L- ARABINOSE	MALTOSE	XYLITOL	A- HYDROXYBUTYRIC ACID	D- SACCHARIC ACID	L- GLUTAMIC ACID	L- THREONINE	D,L-A- GLYCEROL PHOSPHATE	D- ARABITOL	D- MANNITOL	METHYL PYRUVATE	B- HYDROXYBUTYRIC ACID	SEBACIC ACID	GLYCYL-L-ASPARTIC ACID	CARNITINE	GLUCOSE-1-PHOSPHATE	D- CELLOBIOSE	D- MANNOSE	METHYL SUCCINATE	G- HYDROXYBUTYRIC ACID	SUCCINIC ACID	GLYCYL-L-GLUTAMIC ACID	G- AMINO BUTYRIC ACID	GLUCOSE-6-PHOSPHATE
27	0	1	0	1	0	1	1	0	0	0	0	0	1	1	1	0	0	0	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	0	1	1	0	1	0	0	1	1	0	1	1	1	0
28	0	1	1	1	1	1	0	1	1	0	0	1	1	1	0	0	1	0	0	1	1	1	1	1	0	0	0	1	1	0	0	1	1	1	1	1	0	0	1	0	0	1	0	0	1	1	0
29	0	1	1	1	1	1	0	1	1	0	1	1	1	1	0	0	1	0	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	0	0	1	0	0	1	1	0	1	1	1	0
30	0	1	1	1	1	1	0	1	1	0	0	1	1	1	0	0	1	0	0	1	1	1	1	1	0	0	0	1	1	0	0	1	1	1	1	0	0	1	0	0	1	0	0	1	1	0	0
31	0	1	0	1	0	1	1	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	1	0	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	0
32	0	1	1	1	1	1	0	1	1	0	0	1	1	1	0	0	1	0	0	1	1	1	1	1	0	0	0	1	1	0	0	1	1	1	0	0	1	0	0	1	0	0	1	1	1	1	0
33	0	1	0	1	0	0	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	1	1	1	0	0	0	1	1	0	0	0	0	1	0	1	0	0	1	0	0	0	0	0	1	0	1	0
34	0	1	0	1	0	1	1	1	1	0	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	0	0	1	1	1	1	1	0	1	1	1	1	0	0	1	0	0	1	1	0	1	0	1	0
36	0	1	0	1	0	1	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	1	1	1	1	1	1	0	0	0	1	1	0	0	1	1	1	1	0	0	1	0	0	1	1	0	1	0	1	0
38	0	1	0	1	0	1	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	1	1	0	0	1	1	1	0	0	1	0	0	0	1	0	1	0	1	0
38.1	0	1	0	1	0	1	1	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0	1	1	1	1	0	1	1	1	0	0	1	0	0	0	1	0	1	1	1	0
39	0	1	0	1	0	1	1	0	1	0	1	1	1	1	1	0	0	0	0	0	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	0	1	1	0	1	0	0	1	0	0	1	1	1	1	0
41	0	1	0	1	0	1	1	0	0	0	1	0	0	1	1	0	1	0	0	0	1	1	1	1	0	0	0	0	0	1	1	0	1	0	1	0	0	1	0	0	1	0	0	1	1	0	1	0	1	0
42	0	1	0	1	1	1	1	1	1	0	1	1	1	1	1	0	1	0	0	0	1	1	1	1	0	0	1	1	0	1	1	1	1	1	1	1	0	0	1	0	0	1	1	0	1	1	1	0	1	0
43	0	0	0	1	0	1	1	1	0	0	0	0	1	1	1	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	1	0	0
44	0	1	0	1	0	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	1	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0	1	0	0
45	0	1	0	1	0	1	1	0	0	0	0	0	1	1	0	0	0	0	1	0	0	1	0	1	1	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1	1	1	0	0
46.1	0	0	0	1	0	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0	1	0	1	0
47	0	1	1	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	1	1	1	0	0	1	1	1	1	0	0	0	1	1	0	1	0	1	0	0
48	0	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
50	0	1	0	1	1	1	1	1	1	0	0	1	1	1	0	1	0	0	1	0	0	1	1	1	1	1	1	0	0	0	1	1	0	1	1	1	1	0	1	0	0	1	0	0	1	1	0	1	1	0
51	0	1	0	1	0	1	1	0	0	0	0	1	1	1	1	0	0	0	0	0	0	1	1	1	1	1	1	0	0	0	1	1	1	1	1	0	1	1	1	1	1	0	0	1	1	0	1	1	1	0
52	0	1	0	1	0	1	1	0	1	0	1	0	1	1	0	0	1	0	0	0	1	1	1	1	0	0	0	0	0	1	0	0	0	1	1	1	1	0	1	0	0	1	1	0	1	1	1	1	0	0
53	0	1	0	1	1	1	0	1	0	0	0	0	1	1	1	0	1	1	0	0	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	0	1	1	1	1	1	0	0	1	1	0	1	0	1	0
53.1	0	1	0	1	1	0	0	1	0	0	0	0	1	1	1	0	0	1	0	0	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	0	1	1	1	1	1	0	0	1	0	0	1	1	0	1	0
54	0	1	1	1	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	0	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	0	1	1	1	1	1	0	0	1	0	0	1	1	0	1	0
55	0	1	1	1	1	1	1	1	1	0	0	1	1	1	0	1	0	0	0	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	0	0	1	0	0	1	1	0	1	1
56	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	1	0	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	0	1	1	1	1	0	1	0
57	0	0	0	1	1	1	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	0	1	1	0	0	1	1	0	1	1	1	0
58	1	0	1	0	0	1	0	0	1	1	1	0	1	1	0	0	0	0	1	1	0	0	0	1	0	0	1	0	1	0	1	0	0	1	0	0	0	1	1	0	1	0	0	1	1	1	0	1	0	0	0
59	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	0	1	0	0	1	1	1	1	1	1	0
61	1	1	0	1	1	1	0	0	1	0	1	1	0	1	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	0	1	1	1	1	1	1
65	0	1	0	1	1	1	1	0	1	0	1	1	1	1	1	0	1	0	0	0	0	1	1	1	0	0	0	0	0	1	1	0	0	0	1	1	0	0	1	0	0	1	1	0	1	1	1	1	1	0
66	0	1	0	1	0	1	1	1	1	0	1	1	1	1	0	0	1	0	1	0	1	1	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	0	0	1	1	0	1	1	1	1	1	1
67	0	0	0	1	0	1	1	1	0	0	1	0	0	1	0	0	1	0	0	0	0	1	1	1	0	0	0	0	0	0	1	0	1	1	0	0	1	0	0	0	0	0	1	1	0	1	1	0	0	0
68	0	0	0	1	1	0	1	1	1	0	0	0	1	1	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0	0	1	0	0	1	1	1	0	0	0	0	0	0	1	0	0	1	1	1	0
69	0	0	0	1	0	1	1	0	1	0	1	1	0	1	0	0	1	0	0	0	1	1	1	1	0	0	0	0	0	1	0	1	0	0	1	1	0	1	1	0	1	1	0	1	1	1	1	1	1	0

RAW BIOLOG DATA
(including duplicates)

APPENDIX A

SAMPLE NUMBER	I- ERYTHRITOL	D- MELIBIOSE	ACETIC ACID	P- HYDROXYPHENYL ACETIC ACID	BROMO SUCCINIC ACID	L- HISTIDINE	UROCANIC ACID	A- CYCLODEXTRIN	D- FRUCTOSE	B- METHYL-D-GLUCOSIDE	CIS- ACONITIC ACID	ITACONIC ACID	SUCCINAMIC ACID	HYDROXY-L-PROLINE	INOSINE	DEXTRIN	L- FUCOSE	D- PSICOSE	CITRIC ACID	A- KETO-BUTYRIC ACID	GLUCURONAMIDE	L- LEUCINE	URIDINE	GLYCOGEN	D- GALACTOSE	D- RAFFINOSE	FORMIC ACID	A- KETO-GLUTARIC ACID	ALANINAMIDE	L- ORNITHINE	THYMIDINE	TWEEN 40	GENTIOBIOSE	L- RHAMNOSE	D- GALACTONIC ACID LACTONE	A- KETO-VALERIC ACID	D- ALANINE	L- PHENYLALANINE	PHENYLETHYLAMINE	TWEEN 80	A-D- GLUCOSE	D- SORBITOL	D- GALACTURONIC ACID	D,L- LACTIC ACID	L- ALANINE	L- PROLINE	PUTRESCINE
70	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	0	0	1	1	0	1	1	1	0	1	0	0	1	1	0	0	0	0	1	0	1	0	0	0	1	1	1	1	0
71	0	0	0	0	0	1	1	0	0	1	0	1	0	1	1	1	0	0	0	1	1	0	1	1	1	0	1	0	0	1	1	0	0	1	0	1	0	0	1	1	1	0	1	1	1	0
72	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	1	1	0	0	0	1	1	0	0	1	1	0	1	0	0	1	1	1	0	1	0	1	1	0	1	0	0	1	1	0	0	1	1	1	0
73	0	0	1	1	1	1	1	1	0	1	0	1	1	1	1	1	0	0	0	0	1	1	0	0	1	1	0	1	0	0	1	1	1	0	1	0	1	1	0	1	0	0	1	1	0	0	1	1	1	0
74	0	0	1	1	1	1	1	1	0	1	0	1	0	1	1	1	1	0	0	0	0	1	0	0	1	0	0	1	0	0	1	0	1	0	1	0	1	0	0	1	0	0	1	1	0	0	1	1	1	0
75	0	0	1	1	1	1	1	1	0	1	0	1	0	1	0	1	0	0	0	1	1	1	0	1	0	0	0	0	0	1	1	1	1	0	1	0	0	0	0	1	1	0	0	1	1	1	1	1	1	1
76	0	0	1	0	1	1	1	1	0	1	0	1	0	1	1	1	1	0	1	1	1	0	1	0	0	1	0	1	1	1	1	1	0	1	0	0	0	1	1	1	0	0	1	1	1	0	0	1	1	1
77	0	0	1	1	1	1	1	1	0	1	0	1	0	1	1	1	0	0	1	1	1	0	1	1	0	0	0	1	1	1	1	1	0	1	0	0	0	1	1	0	0	1	1	1	1	0	0	1	1	1
78	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0	0	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1
79	0	0	1	0	1	1	1	0	1	0	1	0	1	1	1	1	0	1	1	1	0	1	0	0	0	0	0	1	1	1	1	1	0	1	0	0	0	1	1	0	0	1	1	1	1	0	0	1	1	1
79.1	0	0	1	0	1	1	1	0	1	0	1	0	1	1	1	0	0	0	1	1	1	0	1	0	0	0	0	0	1	1	1	1	1	0	1	0	1	0	1	1	1	0	1	1	0	0	1	1	1	1
80	0	0	1	0	1	1	0	0	1	0	1	0	1	1	1	0	0	0	1	1	0	0	1	0	0	0	0	0	1	1	1	1	1	0	1	0	0	0	1	1	1	0	1	1	1	0	0	1	1	1
82	0	0	1	0	1	0	1	0	0	0	1	0	1	1	1	1	0	0	0	0	1	1	0	0	1	0	0	0	0	0	1	1	1	1	0	1	0	0	0	0	0	0	0	0	1	1	0	0	1	1
83	0	0	1	0	1	1	1	0	0	1	0	1	0	1	1	1	1	0	1	1	1	1	1	0	0	1	0	1	1	1	1	1	1	1	0	1	0	0	1	1	1	1	1	1	1	0	0	1	1	1
84	0	0	1	0	1	1	1	1	0	1	0	1	0	1	1	1	0	0	0	1	0	0	1	0	0	1	0	1	1	1	1	1	1	0	1	0	0	0	1	1	0	1	0	0	1	1	1	1	1	0
85	0	0	1	0	1	1	1	1	0	1	0	1	0	1	1	1	1	1	0	0	1	0	0	1	0	0	0	0	0	1	1	1	1	0	1	0	0	0	0	1	1	1	0	1	1	0	0	1	1	1
86	0	0	1	1	1	1	1	1	0	1	0	1	0	1	1	1	1	0	0	1	1	0	1	1	1	1	0	1	0	1	1	1	1	0	1	0	1	1	0	1	0	1	0	0	1	1	1	1	1	0
87	0	0	0	0	1	1	1	1	1	0	1	0	1	0	1	1	1	1	0	0	1	1	0	0	1	0	0	0	0	0	1	1	1	1	0	1	0	0	0	1	1	0	0	1	1	0	0	1	1	1
88	0	0	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	0	0	1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	0	1	0	0	0	1	1	0	0	1	1	0	0	1	1	1
88.1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	0	1	0	1	0	1	1	1	1	0	1	0	1	1	0	1	1	1	1	1	0	0	1	1	1
89	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	1	1	0	0	1	1
89.1	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	1	0	1	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	1	1	1	1	1	0
90.1	0	0	0	1	1	1	1	0	1	0	1	0	1	1	1	0	0	0	1	0	1	1	0	0	1	0	0	1	0	1	0	1	0	0	1	0	1	0	1	0	1	1	1	0	1	1	1	1	1	1
91	0	0	1	0	1	1	1	0	0	0	1	1	1	1	1	0	0	0	1	1	1	1	1	0	1	0	0	1	0	0	0	1	0	0	1	0	1	0	0	1	1	1	1	1	1	1	1	0	1	0
91.1	1	0	1	0	1	1	1	0	1	0	1	1	1	1	1	0	0	1	1	0	1	1	1	0	1	0	0	1	0	0	0	1	0	0	1	0	1	0	1	0	0	0	1	1	1	1	1	1	1	0
92	0	0	1	1	1	1	1	0	0	0	0	1	1	0	1	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	1	0	1	0	1	0	0	0	0	1	0	1	0	1	0
93	0	0	1	0	1	1	1	0	0	0	1	0	1	1	1	0	0	0	1	1	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	0	1	1	1	0	1	0	0	0	1	1	1	1	1	1
93.1	0	0	0	0	0	1	0	0	0	0	0	1	1	0	1	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	1	0	1	0	0	1	0	0	1	0	0	0	0	1	1	1	0
94	0	0	0	0	1	1	1	1	0	1	0	1	0	0	1	1	0	0	0	0	1	0	0	1	0	0	0	0	0	1	1	1	1	0	1	0	0	0	1	1	0	1	1	1	0	0	1	1	1	1
95	0	0	0	0	1	1	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0
96	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
97	0	0	0	1	1	1	1	1	0	0	0	1	0	1	0	1	0	0	0	1	1	0	1	0	0	0	0	0	1	0	1	1	0	1	0	0	0	1	1	1	1	1	0	0	0	1	1	1	1	0
98	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
99	0	1	1	1	1	0	0	1	1	1	1	0	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	0	0	1	1	1	1	1	1	1	1	1
100.1	0	0	1	0	1	1	0	1	0	0	0	0	0	0	1	1	0	0	0	1	0	1	1	1	0	0	0	1	1	1	1	1	0	0	0	1	1	1	1	1	1	0	1	1	1	1	0	1	1	0
101	1	0	1	1	1	1	1	0	1	0	1	1	1	0	1	0	0	0	1	1	1	1	1	1	0	1	0	1	1	1	1	1	0	1	0	0	0	1	1	1	1	0	1	1	1	1	1	1	1	0
102	0	0	0	0	1	1	1	0	0	0	1	0	1	1	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	0	1	0	0	0	1	1	1	0	1	0
102.1	0	0	0	0	1	0	1	0	0	0	1	0	1	1	0	0	0	1	0	1	0	1	1	0	0	0	0	1	0	1	0	1	0	1	1	0	0	1	0	0	0	1	1	1	0	1	1	1	1	0
103	0	0	1	0	1	1	0	0	1	0	1	0	1	1	1	0	0	1	1	0	1	1	0	0	0	0	1	1	0	1	0	1	0	1	0	0	0	0	1	0	1	1	1	0	1	1	1	1	1	1

RAW BIOLOG DATA
(including duplicates)

APPENDIX A

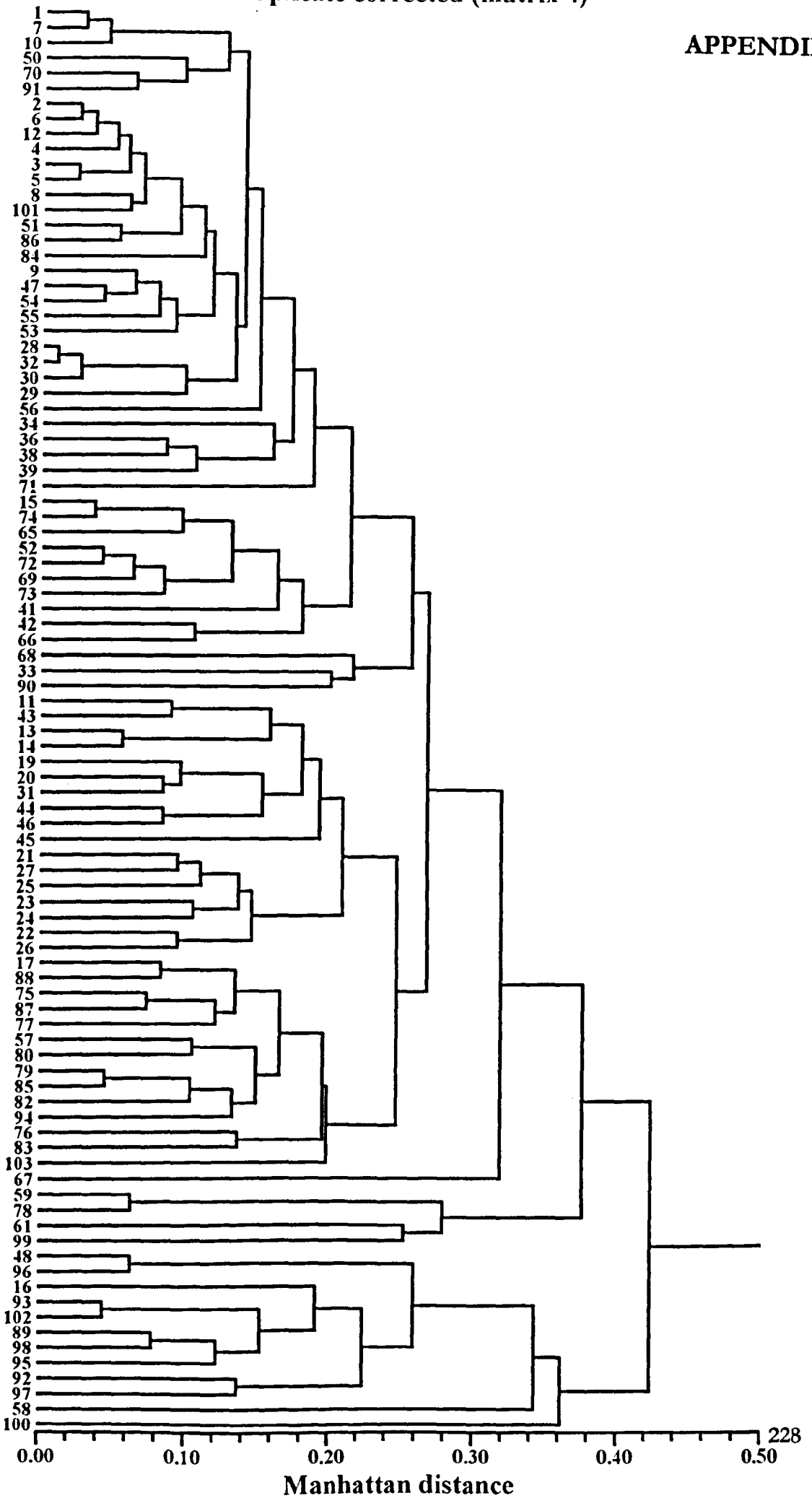
SAMPLE NUMBER	N- ACETYL GALACTOSAMINE	M- INOSITOL	SUCROSE	D- GLUCONIC ACID	MALONIC ACID	L- ALANYL-GLYCINE	L- PYROGLUTAMIC ACID	2- AMINO ETHANOL	N- ACETYL-D-GLUCOSAMINE	A-D- LACTOSE	D- TREHALOSE	D- GLUCOSAMINIC ACID	PROPIONIC ACID	L- ASPARAGINE	D- SERINE	2,3- BUTANEDIOL	ADONITOL	LACTULOSE	TURANOSE	D- GLUCURONIC ACID	QUINIC ACID	L- ASPARTIC ACID	L- SERINE	GLYCEROL	L- ARABINOSE	MALTOSE	XYLITOL	A- HYDROXYBUTYRIC ACID	D- SACCHARIC ACID	L- GLUTAMIC ACID	L- THREONINE	D,L-A- GLYCEROL PHOSPHATE	D- ARABITOL	D- MANNITOL	METHYL PYRUVATE	B- HYDROXYBUTYRIC ACID	SEBACIC ACID	GLYCYL-L-ASPARTIC ACID	CARNITINE	GLUCOSE-1-PHOSPHATE	D- CELLOBIOSE	D- MANNOSE	METHYL SUCCINATE	G- HYDROXYBUTYRIC ACID	SUCCINIC ACID	GLYCYL-L-GLUTAMIC ACID	G- AMINO BUTYRIC ACID	GLUCOSE-6-PHOSPHATE
70	0	1	1	1	1	1	1	1	0	0	1	1	1	1	0	0	1	0	0	1	1	1	1	1	1	0	0	1	1	1	0	1	1	1	1	1	0	0	1	0	0	1	1	1	1	0
71	0	1	0	1	1	1	1	0	1	0	1	1	1	1	0	0	1	0	0	0	1	1	1	1	1	0	1	0	1	1	1	0	1	1	1	1	1	0	0	1	0	0	1	1	0	1	0
72	0	1	0	1	0	1	1	0	1	0	1	1	1	1	0	0	1	0	0	0	0	1	1	1	1	0	0	0	0	0	1	0	0	1	1	1	1	1	0	1	0	0	1	1	0	1	1	1	0
73	0	1	0	1	1	1	1	0	1	0	1	1	1	1	0	0	1	0	0	0	0	0	1	1	1	0	0	0	0	1	0	1	0	1	1	1	1	1	1	0	1	0	0	1	1	0	1	1	0
74	0	1	0	1	1	1	1	1	1	0	1	1	1	1	1	0	1	0	0	0	0	1	1	1	1	0	0	0	0	0	1	0	0	1	1	1	1	1	1	0	0	1	0	0	1	1	0	1	1	0
75	0	0	0	1	0	1	1	1	0	0	0	0	1	1	1	0	0	0	0	0	1	1	1	1	0	0	0	1	0	1	1	0	0	0	0	1	1	0	0	1	0	0	0	0	1	1	1	1	1	0
76	0	0	0	1	1	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	1	1	1	1	1	0	0	1	1	1	1	1	0	0	0	0	1	1	0	0	1	0	0	1	1	1	1	1	0	0
77	0	0	0	1	0	1	1	1	0	0	0	0	1	1	1	0	1	0	0	0	1	1	1	1	1	0	0	0	0	1	1	0	1	0	1	1	0	0	0	0	0	1	0	1	0	1	0	1	0	0
78	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	0	0	1	1	1	1	1	1	1	1
79	0	0	0	1	1	1	1	1	0	0	0	0	1	1	1	0	0	0	1	0	1	1	1	1	0	0	0	1	1	1	1	0	0	1	1	1	0	0	0	1	0	0	1	1	1	1	1	1	0	0
79.1	0	0	0	1	1	1	1	1	0	0	0	1	1	1	1	0	0	0	1	0	1	1	1	1	0	0	0	1	1	1	1	0	0	1	1	1	0	1	1	0	0	1	1	0	1	1	1	1	0	0
80	0	1	1	1	0	1	1	1	1	0	1	1	1	1	0	0	0	0	0	0	1	1	1	1	0	0	0	0	1	1	1	0	1	1	1	1	0	0	1	0	0	1	1	0	1	1	1	1	0	0
82	0	0	0	1	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	0	1	1	1	1	0	0	0	0	1	1	1	0	0	0	0	1	1	0	0	1	0	0	1	1	0	1	1	1	0	0
83	0	0	1	1	1	1	1	1	1	0	0	0	1	1	0	0	0	0	1	1	1	1	1	1	1	0	0	1	1	1	1	0	1	1	1	1	0	0	1	0	0	1	1	1	1	1	1	1	0	0
84	0	1	1	1	1	1	1	1	0	0	1	1	1	1	0	0	0	0	0	1	1	1	1	1	1	0	0	0	1	1	1	0	1	1	1	1	0	0	1	0	0	1	1	0	1	1	1	1	0	0
85	0	0	0	1	1	1	1	1	0	0	0	0	1	1	0	0	0	0	0	1	0	1	1	1	1	0	0	0	0	1	1	1	0	0	1	1	1	0	1	1	0	0	1	1	0	1	1	1	0	0
86	0	1	0	1	1	1	1	1	1	0	0	1	1	1	1	0	0	0	0	0	0	1	1	1	1	1	1	0	0	1	1	1	1	0	1	1	1	1	0	0	1	0	0	1	1	0	1	1	1	0
87	0	0	0	1	0	0	1	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	1	0	0	0	0	1	1	0	0	1	0	0	0	1	1	1	1	1	0
88	0	0	0	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	0	0	0	1	1	1	1	1	1	0	0	0	0	0	1	0	0	0	1	1	0	0	1	0	0	0	1	0	1	1	1	0
88.1	0	0	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	0	0	0	0	1	0	1	1	1	1	1	0	0	0	0	0	1	1	0	0	1	1	1	1	0	0	1	0	1	0	1	1	0
89	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0
89.1	0	0	0	0	0	0	0	1	1	0	0	0	0	1	1	1	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1	1	1	0	1	0	1	0	0	1	0	1	0	0
90.1	0	1	0	1	1	0	1	0	1	0	1	0	1	1	0	0	0	0	0	0	1	1	1	1	1	1	0	0	0	1	1	0	0	0	0	1	1	1	0	0	1	0	0	1	0	0	1	1	0	1	0
91	0	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0	1	0	0	0	1	1	1	1	1	1	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	0
91.1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0	1	0	0	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1	1	0	0	1	0	0	1	1	0	1	0	1	1
92	0	0	0	1	0	1	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	1	1	0	0	0	0	1	1	1	0	0	0	0	0	0	1	1	1	1	0	0
93	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	1	1	1	0
93.1	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	1	0
94	0	0	0	1	0	1	1	1	1	0	0	0	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0	1	1	1	1	0	1	1	1	0	0	1	0	0	1	0	0	1	1	1	0	0
95	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	1	0	1	0	0	0
96	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
97	0	0	0	0	1	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	1	1	0	0	0	0	1	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	1	1	1	1	0	0
98	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0
99	1	1	1	1	0	1	0	1	1	1	1	1	1	1	1	0	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1
100.1	0	0	1	0	0	1	1	0	0	0	0	0	1	1	0	1	0	0	0	0	0	1	1	0	0	1	0	1	1	1	1	1	0	0	0	1	0	0	1	0	1	0	1	1	1	0	1	0	1	1
101	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	0	0	1	1	1	1	1	1	1	0	0	1	1	1	1	0	1	1	1	1	0	0	1	0	0	1	1	0	1	1	1	0	0
102	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	1	0
102.1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	1	0	1	0	0	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
103	0	0	0	1	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0	1	1	1	1	1	1	0	0	0	1	1	0	0	0	0	0	0	1	0	1	1	0	0	1	1	0	1	1	1	1	0

Spoilage Pseudomonas - UPGMA

Duplicate corrected (matrix 4)

APPENDIX B

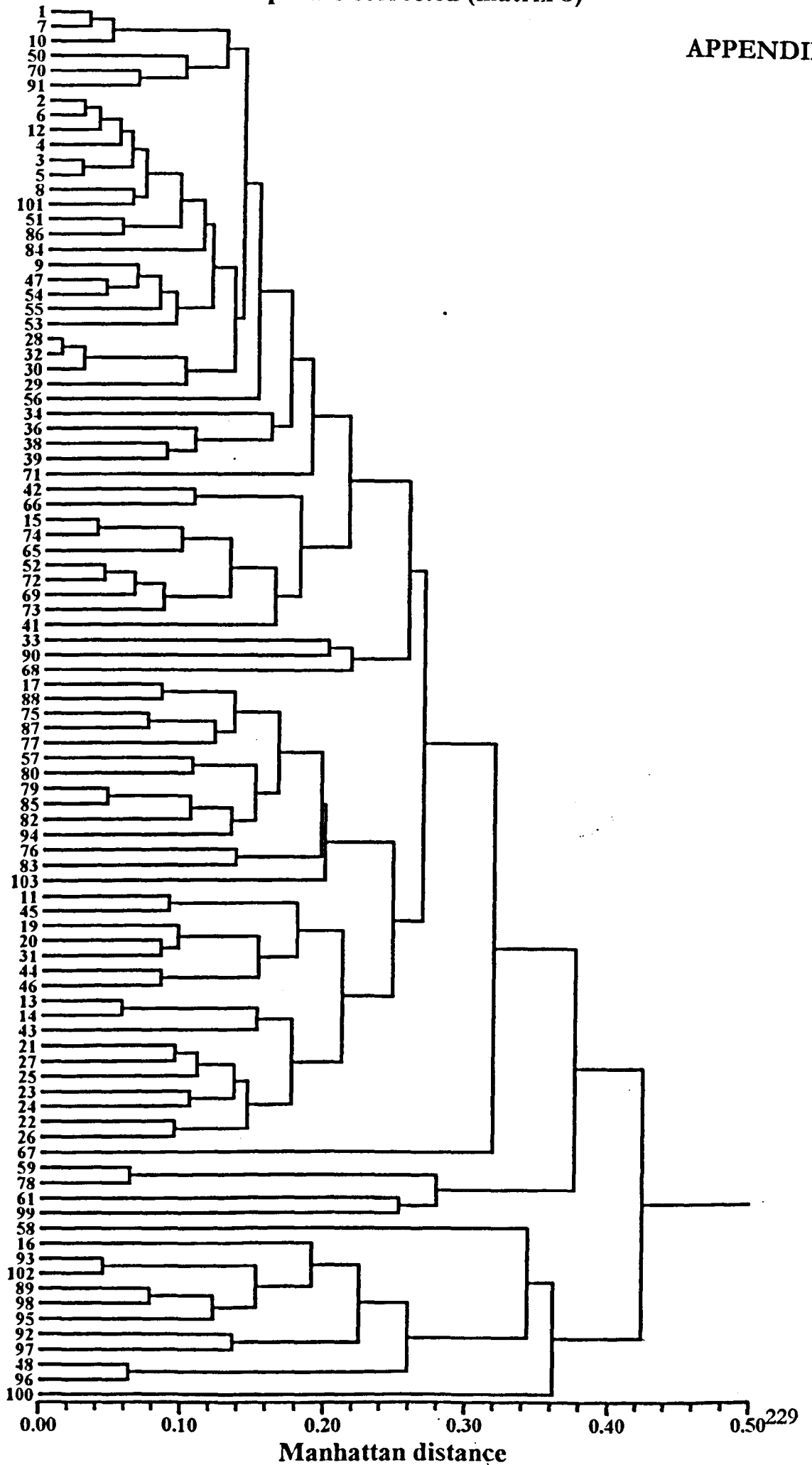
B.1



Spoilage Pseudomonas - UPGMA
Duplicate corrected (matrix 8)

APPENDIX B

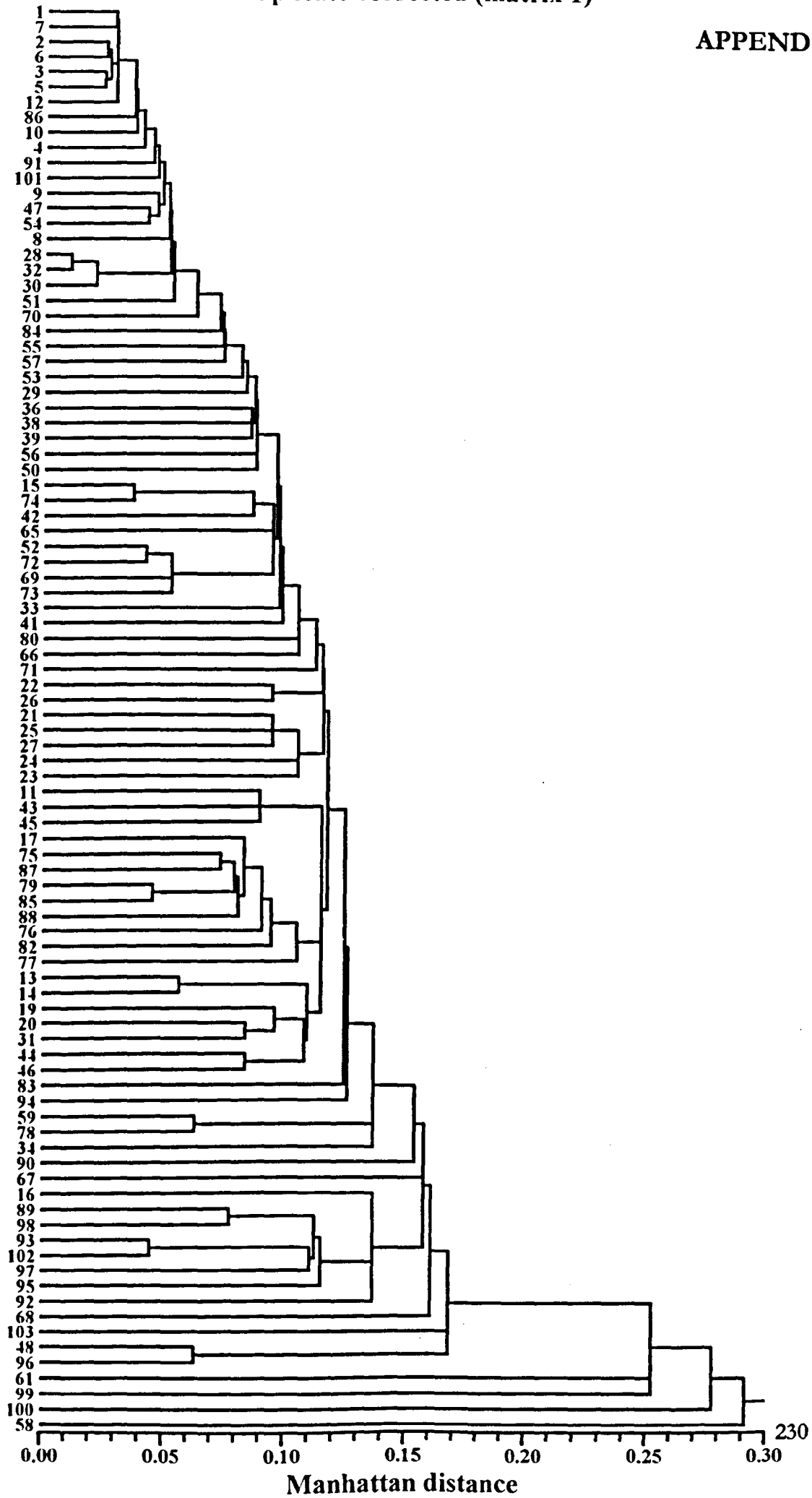
B.2



Spoilage Pseudomonas - Single Linkage
Duplicate corrected (matrix 1)

APPENDIX B

B.3

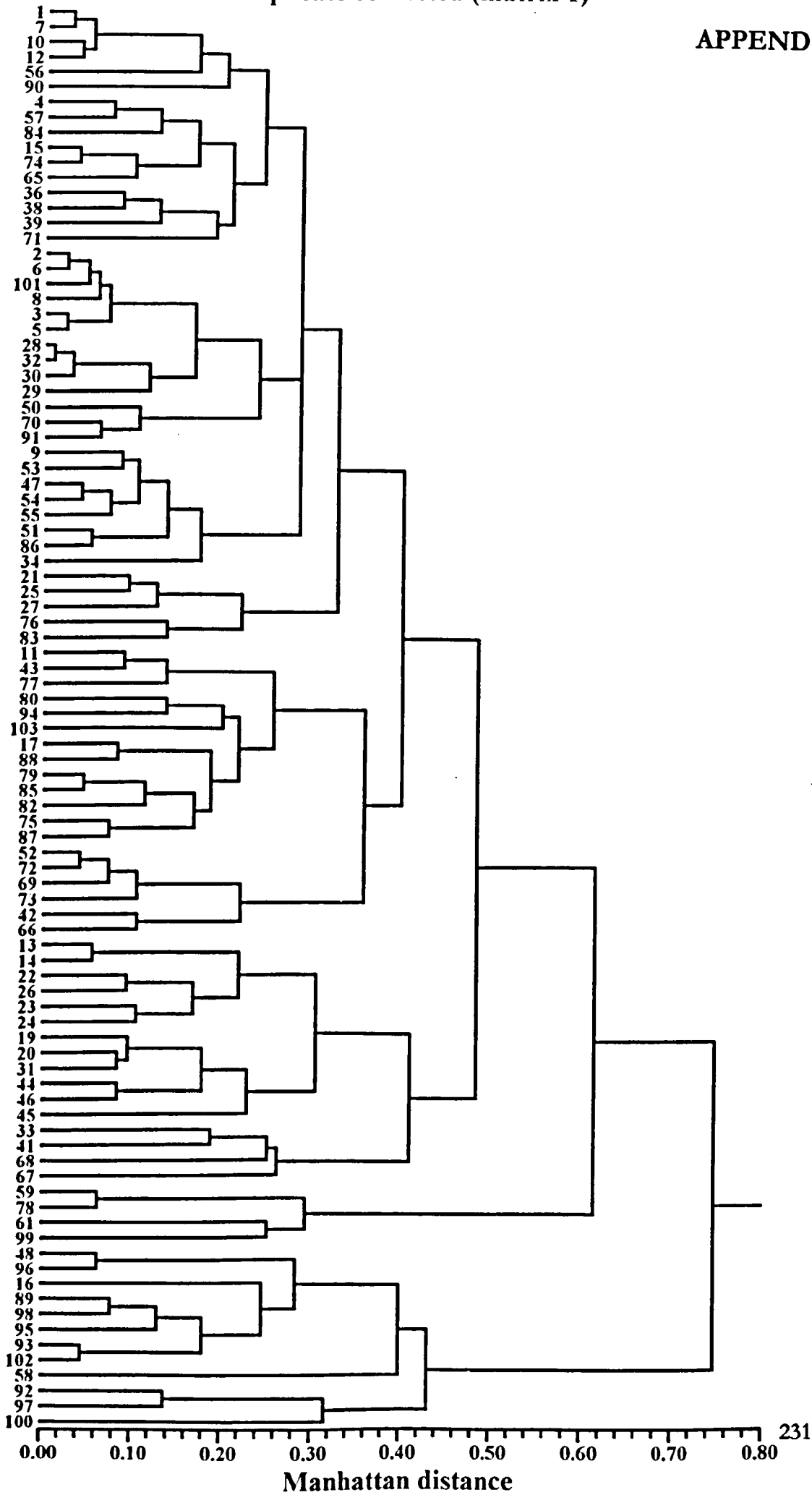


Spoilage Pseudomonas - Complete Linkage

Duplicate corrected (matrix 1)

APPENDIX B

B.4

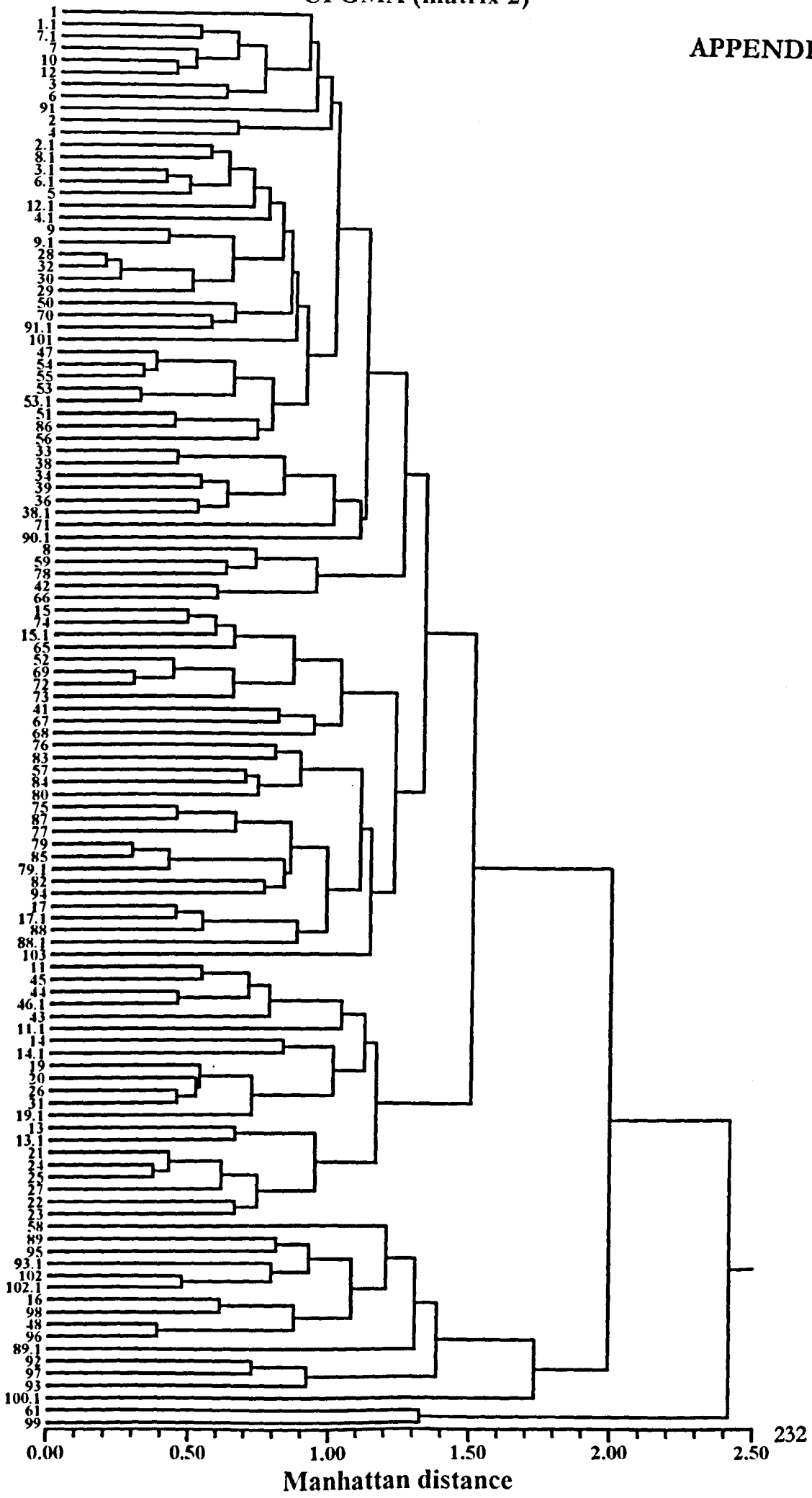


Spoilage Pseudomonas - qualitative

UPGMA (matrix 2)

APPENDIX B

B.5



REFERENCES

ALCOCK, S. C., CHAFFEY, B. J., DODD, C. E. R., MORGAN, M. R. A. and WAITES, W. M. (1992) Applications of immunoassay systems. In: *Food Safety and Quality Assurance.* M. R. A. Morgan, C. J. Smith, and P. A. Williams (Eds.) pp 459-466. Elsevier.

ALDERSON, G. (1985) The application and relevance of nonhierarchic methods in bacterial taxonomy. In: *Computer-assisted bacterial systematics.* M. Goodfellow, D. Jones & F. G. Priest (Eds). pp 227-263. Academic Press Inc., London.

ALTWEGG, M. & MAYER, L. W. (1989) Bacterial molecular epidemiology based on a non-radioactive probe complementary to ribosomal-RNA. *Research in Microbiology* 140(4-5):325-333.

ANON. (1998) Molecular probes, Inc. homepage. http://www.probes.com.

ANON. (2000). http://www.antibodyresource.com.

ANWAR, H. M., DASGUPTA, M. K., LAM, K. & COSTERTON, J. W. (1989) Tobramycin resistance of mucoid *Pseudomonas aeruginosa* biofilm grown under iron limitation. *Journal of Antimicrobial Chemotherapy* 24:647-655.

ARNAUTROLIER, I., VAUTERIN, L., DE VOS, P., MASSART, D. L., DEVRIESE, L. A., DE ZUTTER, L. & VAN HOOF, L. (1999) A numerical taxonomy study of the *Pseudomonas* flora isolated from poultry meat. *J. Appl. Microbiol.* 87 (1):15-28.

AUSUBEL, F. M., BRENT, R. & KINGSTON, R. E. (1990) *Short protocols in molecular biology: a compendium of methods from current protocols in molecular biology.* Greene Publishing and Wiley-Interscience, New York.

BASCOMB, S. (1989) Computers in taxonomy and systematics. In: *Computers in microbiology. A practical approach.* T. N. Bryan & J. W. T. Wimpenny (Eds) pp 65-102. IRL Press, Oxford.

BERGEY'S MANUAL OF DETERMINATIVE BACTERIOLOGY (1957) 7TH edition. S. Robert, E. G. Breed, D. Murray, R. Nathan-Smith (Eds). Ballière, Tindall & Cox, London.

BERGEY'S MANUAL OF DETERMINATIVE BACTERIOLOGY (1974) 8TH edition. R. E. Buchanan & N. E. Gibbons (Eds). Williams & Wilkins, Baltimore.

BERGEY'S MANUAL OF SYSTEMATIC BACTERIOLOGY (1989) Volume 1. J. G. Holt (Editor-in-chief). Williams & Wilkins, Baltimore.

BINGEN, E. H., DENAMUR, E. & ELION, J. (1994) Use of ribotyping in epidemiological surveillance of nosocomial outbreaks. *Clin. Microbiol. Rev.* 7:311-327.

BLANC, D. S., SIEGRIST, H. H., SAHLI, R., & FRANCIOLI, P. (1993) Ribotyping of *Pseudomonas-aeruginosa* - discriminatory power and usefulness as a tool for epidemiological studies. *Journal of Clinical Microbiology* 31(1):71-77.

BLENKINSOPP, S. A. & COSTERTON, J.W. (1991). Understanding bacterial biofilms *Trends in Biotechnology* 9:138-143.

BLOEMBURG, G. V., O'TOOLE, G. A., LUGTENBERG, B. J. J. & KOLTER, R. (1997) Green fluorescent protein as a marker for *Pseudomonas* spp. *Appl. Environ. Microbiol.* pp 4543-4551.

BOCHNER, B. R. (1989) Sleuthing out bacterial identities. *Nature.* 339:157-158.

BODDY, L. & WIMPENNY, J. W. T. (1992) Ecological concepts in food microbiology. *Journal of Applied Bacteriology* 73(SS):S23-S38.

BOYCE, A. J. (1969) Mapping diversity: a comparative study of some numerical methods. In: *Numerical taxonomy.* A. J. Cole (Ed) pp 1-31. Academic Press, London.

BRIDSON, E. Y. (1990) The Oxoid manual, Unipath, Basingstoke, UK.

BRISOU, J. (1958) Etude de quelques Pseudomonadaceae. Bordeaux: A. Baillet.

BROCK, T. D. & MADIGAN, M. T. (1988) Biology of microorganisms. 5th edition. Prentice Hall International, Inc.

BROCKLEHURST, T. F., PARKER, M. L., GUNNING, P.A., COLEMAN, H. P. & ROBINS, M. M. (1995) Growth of food-borne pathogenic bacteria in oil-in-water emulsions: II -Effect of emulsion structure on growth parameters and form of growth. *Journal of Applied Bacteriology* 78:609-615.

BROSCH, R., LEFEVRE, M., GRIMONT, F. & GRIMONT, P. (1996) Taxonomic diversity of pseudomonads revealed by computer-interpretation of ribotyping data. *System. Appl. Microbiol.* 19:541-555.

CHAMBERS, S. J., WYATT, G. M., GARRETT, S. D. & MORGAN, M. R. A. (1999) Alteration of the binding characteristics of a recombinant scFv anti-parathion antibody - 2. Computer modelling of hapten docking and correlation with ELISA binding. *Food and Agricultural Immunology* 11(3):219-228.

CLIFFORD, H. T. & STEPHENSON, W. (1975) *An introduction to numerical classification.* Academic Press Inc., London.

COONS, A. H. & KAPLAN, M. H. (1950) Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen by means of fluorescent antibody. *J. Exp. Med.* 91:1-13.

COONS, A. H., CREECH, H. J. & JONES, R. N. (1941) Immunological properties of an antibody containing a fluorescent group. *Proc. Soc. Exp. Biol.* 47:200-202.

COONS, A. H., CREECH, H. J., JONES, R. N. & BERLINER, E. (1942) The demonstration of pneumococcal antigen in tissues by the use of fluorescent antibody. *J. Immunol.* 45:159-170.

COSTAS, M., HOLMES, B., ON, S. L. W. & STEAD, D. E.(1992) Identification of medically important *Pseudomonas* species using computerized methods. In: *Identification methods in applied and environmental microbiology.* R. G Board, D. Jones, and F. A Skinner (Eds). pp 1- 20. Blackwell Scientific Publications.

COUSIN, M. A. (1982) Presence and activity of psychrotrophic microorganisms in milk and dairy-products: A review. *Journal of Food Protection* 45(2):172-207.

COWAN & STEEL's manual for the identification of medical bacteria (1974) 2nd edition revised by S.T. Cowan. Cambridge University Press.

DAVEY, B. (1992) *Immunology a foundation text.* John Wiley & Sons, Chichester.

DE VOS, P. & DE LEY, J. (1983) Intra- and intergeneric similarities of *Pseudomonas* and *Xanthomonas* ribosomal ribonucleic acid cistrons. *Int. J. Syst. Bacteriol.* 33:487-509.

DE VOS, P., GOOR, M., GILLIS, M. & DE LEY, J. (1985) Ribosomal ribonucleic acid cistron similarities of phytopathogenic *Pseudomonas* species. *Int. J. Syst. Bacteriol.* 35:169-184.

DE VOS, P., VAN LANDSCHOOT, A., SEGERS, P., TYYGAT, R., GILLIS, M., BAUWENS, M., ROSSAU, R., GOOR, M., POT, B., KERSTERS, K., LIZZARAGA, P. & DE LEY, J. (1989) Genotypic relationships and taxonomic localization of unclassified *Pseudomonas* and *Pseudomonas*-like strains by deoxyribonucleic acid: ribosomal ribonucleic acid hybridizations. *Int. J. Syst. Bacteriol.* 39:35-49.

DEN DOOREN DE JONG, L. E. (1926) Bijdrage tot de kennis van het mineralisatieprocess. Nijgh & Van Ditmar, Rotterdam pp 1-200.

DHIR, V. K. & DODD, C. E. R. (1995) Susceptibility of suspended and surface-attached *Salmonella enteritidis* to biocides and elevated temperatures. *Applied and Environmental Microbiology* 61:1731-1738.

DODD, C. E. R. & DAINTY, R. H. (1992) Identification of *Brochothrix* by intracellular and surface biochemical composition. In *Identification Methods in Applied and Environmental Microbiology.* R. G. Board D. Jones & F. A. Skinner (eds.) pp 297-310. Blackwell Scientific.

DODD, C. E. R. & WAITES, W.M. (1991). The use of toluidine blue for *in situ* detection of micro-organisms in foods. *Letters in Applied Microbiology* 13:220-223.

DODD, C. E. R. & WAITES, W. M. (1992) Development of microscopic techniques for examining microbial growth *in situ* in food ecosystems. In: *Ecosystems: Microbes: Food.* R. G. Board, D. Jones, R. G Kroll and G. L.Pettipher (Eds.) 21:1148-1154. *Society for Applied Bacteriology Symposium Series.*

DON, R. H., COX, P. T., WAINWRIGHT, B. J., BAKER, K. & MATTICK, J. S. (1991) Touchdown PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Research* 19(14):4008.

DOYLE, M. P., BEUCHAT, L. R. & MONTVILLE, T. J. (1997) *Food microbiology: fundamentals and frontiers.* ASM Press, Washington DC.

DUNN, G. & EVERITT, B. S. (1982) *An introduction to mathematical taxonomy. Cambridge studies in mathematical biology.* Cambridge University Press, Cambridge.

EDDY, B. P. (1960) The use of and meaning of the term 'psychrotrophic'. *J. Appl. Bacteriol.* 23:189-190.

FAIRBURN, D. J. & LAW, B. A. (1986) Proteinases of psychrotrophic bacteria: their production, properties, effects and control. *Journal of Dairy Research* 53:139-177.

FAUDE, U. C. & HOFLE, M. G. (1997) Development and application of monoclonal antibodies for *in situ* detection of indigenous bacterial strains in aquatic ecosystems. *Applied and Environmental Microbiology* 63(11):4534-4542.

FERNANDES, M. A., JACKMAN, P. J. H., CLARK, S. A. & GUNNARD, S. R. (1988) Detection and quantification of micro-organisms in a heterogeneous foodstuff by image analysis. *CABIOS* 4:291- 295.

FLINT, F. O. & FIRTH, B. M. (1988) Improved toluidine blue stain mountant for microscopy of food products, *Analyst* 113:365-366.

FOOD EMULSIONS (1997) E. Stig-Friberg & Kare Larssons (Eds). Marcel Dekker Inc.

FRANK, J. F. & KOFFI, R. A. (1990) Surface adherent growth of *Listeria monocytogenes* is associated with increased resistance to surfactant sanitizers and heat. *Journal of Applied Bacteriology* 53:122-124.

GARCIA, M. L., SANZ, B., GARCIACOLLIA, P. & ORDONEZ, J. A. (1989). Activity and thermostability of the extracellular lipases and proteinases from pseudomonads isolated from raw-milk. *Milchwissenschaft-Milk Science International* 44(9):547-549.

GARRETT, S. D., APPLEFORD, D. J. A., WYATT, G. M., LEE, H. A. & MORGAN, M. R. A. (1997) Production of a recombinant anti-parathion antibody (scFv). Stability in methanolic food extracts and comparison to an anti-parathion monoclonal antibody. *Journal of Agricultural and Food Chemistry* 45(10)4183-4189.

GILLIGAN, P. H. (1991). Microbiology of airway disease in patients with cystic-fibrosis *Clinical Microbiology Reviews* 4(1):35-51.

GODING, J. W. (1983) Monoclonal antibodies: Principle and practice. 2nd edition Academic Press.

GOODFELLOW, M. & WILLIAMS, E. (1986) New strategies for the selective isolation of industrially important bacteria. *Biotechnol. Genetic Eng. Rev.* 4:213-262.

GOUNOT, A. M. (1991) Bacterial life at low-temperature - physiological aspects and biotechnological implications. *Journal of Applied Bacteriology* 71(5):386-397.

GRATTARD, F., POZZETTO, B., ROS, A. & GAUDIN, O. G. (1994) Differentiation of *Pseudomonas aeruginosa* strains by ribotyping high discriminatory power by using a single restriction endonuclease. *Journal of Medical Microbiology* 40(4):275-281.

GRIMBERG, S. J., STRINGFELLOW, W. T. & AITKEN, M. D. (1996) Quantifying the biodegradation of phenanthrene by *Pseudomonas stutzeri* P16 in the presence of a nonionic surfactant. *Applied and Environmental Microbiology* 62(7):2387-2392.

GRIMONT, F. & GRIMONT, P. (1986) Ribosomal ribonucleic acid gene restriction patterns as possible taxonomic tools. *Ann. Inst. Paster/Microbiol.* (Paris) 137b:165-175.

GRIMONT, P. A. D., VANCANNEYT, M. L., VANDEMEULEBROECKE, L. V., VAUTERIN, L., BROSCH, R., KERSTERS, K. & GRIMONT, F. (1996) Ability of Biolog and Biotype-100 systems to reveal the taxonomic diversity of the pseudomonads. *System. Appl. Microbiol.* 19:510-527.

GUERIN, W. F. & BOYD, S. A. (1995) Maintenance and induction of naphthalene degradation activity in *Pseudomonas-putida* and an *Alcaligenes* spp under different culture conditions. *Applied and Environmental Microbiology* 61(11):4061-4068.

GUNNING, P. A. (1996) Emulsion structure and bacterial growth. MSc thesis, University of Salford.

GUNNING, A. P., WILDE, P. J., CLARK, D. C., MORRIS, V. J., PARKER, M. L., & GUNNING, P. A. (1996) Atomic force microscopy of interfacial protein films. *Journal of Colloid and Interface Science* 183(2):600-602.

HEERTJE, I. (1993) Structure and function of food products: a review. *Food Structure* 12:343-364.

HEYNDRICKX, M., VAUTERIN, L., VANDAMME, P., KERSTERS, K. & DEVOS, P. (1996) Applicability of combined amplified ribosomal DNA restriction analysis (ARDRA) patterns in bacterial phylogeny and taxonomy. *Journal of Microbiological Methods* 26(3):247-259.

HICKS, S. J. & ROWBURY, R. J. (1987) Bacteriophage resistance of attached organisms as a factor in the survival of plasmid-bearing strains of *E.coli*. *Letters in Applied Microbiology* 4:129-132.

HOLAH, J. T., HIGGS, C., ROBINSON, S., WORTHINGTON, D. & SPENCELEY, H. (1990) A conductance-based surface disinfection test for food hygiene. *Letters in Applied Microbiology* 11:255-259.

HOLLOWAY, B. W., KRISHNAPILLAI, V., & MORGAN, A. F. (1979) Chromosomal genetics of *Pseudomonas*. *Microbiological Reviews* 43:73-102.

HOLMBERG, K. & NORD, C. E. (1984) Application of numerical taxonomy to the classification and identification of microaerophilic actinomycetes. In: *Methods in microbiology*. Vol. 16 pp 341-360. T. Bergan. (Ed). Academic Press, London.

HOWARD, B. J. (1994) Clinical and pathogenic microbiology 2nd edition. St. Louis, Mosby.

HUGH, R. & LEIFSON, E. (1953) The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram-negative bacteria. *J. Bact.* 66:24.

INGRAM , M. & SHEWAN, J. M (1960) Introductory reflections on the *Pseudomonas-Achromobacter* group. *J. Appl. Bacteriol.* 23:373-378.

JARDINE, N. & SIBSON, R. (1968) The construction of hierarchic and non-hierarchic classifications. *Computer J.* 11:177-184.

JESSEN, O. (1965) *Pseudomonas aeruginosa* and other green fluorescent pseudomonads. A taxonomic study. Copenhagen, Munksgaard.

JOHNSEN, K., ANDERSEN, S. & JACOBSEN, C. S. (1996) Phenotypic and genotypic characterization of phenanthrene degrading fluorescent *Pseudomonas* biovars. *Applied and Environmental Microbiology* 62(10):3818-3825.

JOHNSON, S. M., PARSONS, E. Y., STRINGER, S. C., DODD, C. E. R., MORGAN, M. R. A. & WAITES, W. M. (1996) Use of antibody-linked probes for the immuno-location of microorganisms in food systems. *Food and agricultural immunology* 8:239-248.

JOHNSON, J. L. & PALLERONI, N. J. (1989) Deoxyribonucleic-acid similarities among *Pseudomonas* species. *International Journal of Systematic Bacteriology* 39(3):230-235.

JONES, D. & SACKIN, M. J. (1980) Numerical methods in the classification and identification of bacteria with especial reference to the Enterobacteriaceae. In: *Microbiological classification and identification.* M. Goodfellow & R. G. Board (eds.) pp 73-106. Academic Press Inc., London.

JONES, J. B., CHASE A. R. & HARRIS, G. K. (1993) Evaluation of the Biolog GN microplate system for identification of some plant pathogenic bacteria. *Plant Disease.* 77:553-558.

KERSTERS, K., LUDWIG, W., VANCANNEYT, M., DE VOS, P., GILLIS, M. & SCHLEIFER, K-H. (1996) Recent changes in the classification of the pseudomonads an overview. *System. Appl. Microbiol.* 19:465-477.

KING, E. O., WARD, M. K. & RANEY, D. E. (1954) Two simple media for the demonstration of pyocyanin and fluorescein. *J. Lab. Clin. Med.* 44:301.

KIRBY, A. R. & MORRIS, V. J. (1994). New microscopes feel their way. *Techniques in Biology* 41:9-13.

KLUYVER, A. J. & VAN NIEL, C. B. (1936) The prospects for a natural system of classification of bacteria. Zbl.Bakt. 94:369.

KOHLER, G. & MILSTEIN, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* 256:495-497.

LAW, B. A., ANDREWS, A. T., CLIFFE, A. J., SHARPE, M. E. & CHAPMAN, H. R. (1979) Effect of proteolytic raw milk psychrotrophs on Cheddar cheese making with stored milk. *J. Dairy Res.* 46:497-510.

LAW, B. A. (1979) Reviews of the progress of dairy science: Enzymes of psychrotrophic bacteria and their effects on milk and milk products. *J. Dairy Res.* 46:573-588.

LAWRENCE, J. R., KORBER, D. R., HOYLE, B. D., COSTERTON, J. W. & CALDWELL, D. E. (1991) Optical sectioning of microbial biofilms. *Journal of Bacteriology* 173(20):6558-6567.

LESSEL, E. F. & HOLT, J. G. (1970) Presenting and interpreting the results. In: *Methods for numerical taxonomy.* W. R. Lockhart & J. Liston (eds.) pp 50-58. American Society for Microbiology, Bethesda, Maryland.

LI, X., DORSCH, M., DEL TOT, T., SLY, L. I., STACKEBRANDT, E. & HAYWARD, A. C. (1993) Phylogenetic studies of the rRNA group II pseudomonads based on 16S rRNA gene sequences. *J. Appl. Bacteriol.* 74:324-329.

LOCKHART, W.R. (1970) Coding the data In: *Methods for numerical taxonomy.* W. R Lockhart. & J. Liston (eds.) pp 22-23. American Society for Microbiology, Bethesda, Maryland.

LOGAN, N. A. & BERKLEY, R. C. W. (1981) Identification of *Bacillus* strains by API tests. *J. Med. Microbiol.* 20:75-85.

LOGAN, N. A. (1994) *Bacterial systematics.* Blackwell Scientific Publications, Oxford.

LUDWIG, J., NEWMANN, W., KLUGHAUER, N., BROCKMANN, C., ROLLER, C., JILG, S., REETZ, K., SCHACHTNER, I., LUDVIGSEN, A., WALLNER, G., BACHLEITNER, M., FISHER, U. & SCHLEIFER, K. H. (1993) Phylogenetic relationships of bacteria based on comparative sequence analysis of elongation fact Tu and ATP-synthase beta subunit genes. *Antonie Leeuwenhoek J. Microbiol. Serol.* 64:285-305.

LUND, B. M. (1992). Ecosystems in vegetable foods. *Journal of Applied Bacteriology* 73:S115-126.

MARCELLINO, S. N. & BENSON, D. R. (1992) Scanning electron and light microscopic study of microbial succession on Bethlehem St. Nectaire cheese. *Applied and Environmental Microbiology* 58:3448-3454.

MCMEEKIN, T. A., OLLEY, J., ROSS, T., & RATKOWSKY, D. A. (1993) Predictive microbiology : theory and application. John Wiley & Sons, New York.

MIGULA, W. (1894) Ueber ein neues System der Bakterien. *Arb. Bakteriol. Inst. Karlsruhe.* 1:235-238.

MILLER, J. M. & RHODEN, D. L. (1991) Preliminary evaluation of Biolog, a carbon source utilization method for bacterial identification. *Journal of Clinical Microbiology* 29:1143-1147.

MILLER, J. M., BIDDLE, J. W., QUENZER, V. K. & MCLAUGHLIN, J. C. (1993) Evaluation of Biolog for identification of members of the family Micrococceae. *Journal of Clinical Microbiology* 31:3170-3173.

MOORE, E. R. B., MAU, E., ARNSCHEIDT, A., BOTTGER. E. C., HUTSON, R. A., COLLINS. M. D., VAN DER PEER, Y., DE WACHTER. R. & TIMMIS, K. N. (1996) The determination and comparison of the 16S rRNA gene sequences of species of the genus *Pseudomonas (sensu stricto)* and estimation of the natural intrageneric relationships. *System Appl. Microbiol.* 19:478-492.

MORAIS, P. V., MESQUITA, C., ANDRADE, J. L. & DA COSTA, M. S. (1997) Investigation of persistent colonization by *Pseudomonas aeruginosa*-like strains in a spring water bottling plant. *Applied and Environmental Microbiology* 63(3):851-856.

MORITA, R. Y. (1975) Psychrophillic bacteria. *Bacteriol. Rev.* 39:144-167.

MOSSEL, D. A. A. & STRUIJK, C. B. (1992). The contribution of microbial ecology to management and monitoring of the safety, quality and acceptability of foods. *Journal of Applied Bacteriology* 73(SS):S1-S22.

MULLIS, K., FALOONA, F., SCHARF, S., SAIKI, R., HORN, G. & ERLICH, H. (1986) Specific enzymatic amplification of DNA *in vitro* - the polymerase chain-reaction. *Cold Spring Harbor Symposia on Quantitative Biology* 51 (1):263-273.

OBRIEN, M. & COLWELL, R. R. (1987) Characterisation tests for numerical taxonomy studies. In *Methods in Microbiology* Volume 19. R. R. Colwell and R. Grigorova (Eds) *Current methods for classification and identification of microorganisms* pp 69-104. Academic Press Inc., London.

ORLA JENSEN, S. (1909) Die Hauptlinien des naturlichen Bakterien-systems. Zbl. Bakt. 22:305.

O'SULLIVAN, D. J. & O'GARA, F. (1992) Traits of fluorescent *Pseudomonas* spp involved in suppression of plant-root pathogens. *Microbiological Reviews* 56(4):662-676.

OWEN, R. J. & HERNANDEZ, J. (1993) Ribotyping and arbitary-primer PCR fingerprinting of *Campylobacter*. In: *New techniques in food and beverage microbiology*. Kroll, R. G. (Ed). Blackwells, pp 265-285.

OYAIZU, H. & KOMAGATA, K. (1983) Grouping of *Pseudomonas* species on the basis of cellular fatty acid composition and the quinone system with special reference to the existence of 3-hydroxy fatty acids. *J. Gen. Appl. Microbiol.* 29:17-40.

PALLERONI, N. J. (1986) Taxonomy of the pseudomonads. *In The bacteria a treatise on structure and function* pp 3-20. J. R. Sokatch and L.N Ornston. (Eds). Williams & Wilkins, London.

PALLERONI, N. J. (1992) Present situation of the taxonomy of the aerobic pseudomonads. In: *Pseudomonas* molecular biology and biotechnology. E. Galli, S. Silver and Withold (Eds) pp 105-115. American society for microbiology, Washington DC.

PALLERONI, N. J., BALLARD, R. W., RALSTON, E. & DOUDOROFF, M. (1972) Deoxyribonucleic acid homologies in the genus *Pseudomonas*. *Int. J. Syst. Bacteriol.* 23:333-339.

PALLERONI, N. J. (1978) *The Pseudomonas group*. Meadowfield Press, Shildon, England.

PALLERONI, N. J. (1984) Genus I *Pseudomonas* 1894 pp 141-199. In: N. R. Krieg and J. G. Holt (Eds) *Bergey's manual of systematic bacteriology*. Volume I. The Williams & Wilkins Co., Baltimore.

PALLERONI, N. J., KUNISAWA, R., CONTOPOULOU, R. & DOUDOROFF, M. (1973) Nucleic acid homologies in the genus *Pseudomonas*. *Int. J. Systematic Bac.* 23:333-339.

PANKHURST, R. J. (1991) *Practical taxonomic computing.* Cambridge University Press, Cambridge.

PARKER, M. L., BROCKLEHURST, T. F., GUNNING, P. A., COLEMAN, H. P. & ROBINS, M. M. (1995) Growth of food-borne pathogenic bacteria in oil-in-water emulsions. 1. Methods for investigating the form of growth. *Journal of Applied Bacteriology* 78(6):601-608.

PEMBERTON, R. M., HART, J. P. & FOULKES, J. A. (1997) Development of a sensitive, selective electrochemical immunoassay for progesterone in cow's milk based on a disposable screen-printed amperometric biosensor. *Electrochimica Acta.* 43:3567-3574.

PETERSON, A. C. & GUNDERSON, M. F. (1960) Some characteristics of proteolytic enzymes from *Pseudomonas fluorescens. Appl. Microbiol.* 8:98-104.

PHILIPS, J. D., GRIFFITHS, M. W. & MUIR, D. D. (1981) Growth and associated enzymic activity of spoilage bacteria in pasteurised double cream. *Journal of the Society of Dairy Technology* 34(3):113-146.

POLLARD-KNIGHT, D. (1990) Current methods in nonradioactive nucleic acid labeling and detection. *Technique* 2:113-132.

PREVOT, A-R. (1933) Etudes des systematique bacterienne. Ann. Sci. Nat. 15:23.

PRIEST, F. & AUSTIN, B. (1993) *Modern bacterial taxonomy.* Chapman & Hall, London.

PRIEST, F. G. & WILLIAMS, S. T. (1993) Computer-assisted identification. In: *Handbook of new bacterial systematics.* M. Goodfellow & A. G. O'Donnell (Eds.) pp 361-381. Academic Press Limited, London.

ROBINS, M., BROCKLEHURST, T. & WILSON, P. (1994) Food structure and the growth of pathogenic bacteria. *Food Technology International Europe* pp 31-36.

ROBINS, M. M. & WILSON, P. D. G. (1994) Food structure and microbial growth. *Trends* In: *Food Science and Technology* 5(9):289-293.

RONCALES, P., AGUILERA, M., BELTRAN, J. A., JAIME, I. & PEIRO, J. M. (1991) The effect of natural or artificial casing on the ripening and sensory quality of a mould-covered dry sausage. *International Journal of Food Science and Technology* 26:83-89.

SACKIN, M. J. & JONES, D. (1993) Computer-assisted classification. In: *Handbook of new bacterial systematics.* M. Goodfellow, & A. G. O'Donnell (Eds.) pp 281-313. Academic Press Limited, London.

SACKIN, M. J. (1987) Computer programs for classification and identification. In: *Methods in microbiology.* Volume 19. R.R. Colwell & R. Grigorova (Eds.) pp 21-36. *Current methods for classification and identification of microorganisms.* Academic Press Inc., London.

SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. (1989) *Molecular cloning: a laboratory manual.* Books 1-3. Cold Spring Harbour Laboratory Press, New York.

SAYLER, G. S., KING, J. M. H., BURLAGE, R. S. & LARIMER, F. (1990) Molecular analysis of biodegradative bacterial population application of bioluminescence technology. *Abstracts of Papers of the American Chemical Society* 199(1):153-ENVR.

SCHERTHAN, H., KIOSCHIS, P. & ZANKI, H. (1990) Application of reflection contrast and fluorescence microscopy on non- radioactive *in situ* hybridisation. *Laboratory Practice* 39:61-64.

SCHMIDT-NIELSON, S. (1902) Ueber einige psychrophile mikroorganismen und ihr vorkommen. *Zbl. Bakt.* (Abt.2) 9:145.

SHAH, N. P. (1994) Psychrotrophs in milk-a review. *Milchwissenschaft-Milk Science International* 49(8):432-437.

SMIT, E., LEEFLANG, P. & WERNARS, K. (1997) Detection of shifts in microbial community structure and diversity in soil caused by copper contamination using amplified ribosomal DNA restriction analysis. *FEMS Microbiology Ecology* **23**(3):249-261.

SNEATH, P. H. A. & JOHNSON, R. (1972) The influence of numerical taxonomic similarities of errors in microbiological tests. *J. Gen. Microbiol.* **24**:508-523.

SNEATH, P. H. A. & SOKAL, R. R. (1973*) Numerical taxonomy.* W. H. Freeman and Company, San Francisco.

SNEATH, P. H. A. (1957a) Some thoughts on bacterial classification. *J. Gen Microbiol.* **17**:184-200.

SNEATH, P. H. A. (1957b) The application of computers to taxonomy. *J. Gen Microbiol.* **17**:201-226.

SNEATH, P. H. A. (1962) The construction of taxonomic groups. In: *Microbial classification.* G. C. Ainsworth & P. H. A. Sneath (Eds) Cambridge University Press, Cambridge, pp 289-332.

SNEATH, P. H. A. (1972) Computer taxonomy In: *Methods in microbiology* Volume 7A Norris, J. R. & Ribbons, D.W. (Eds) pp 29-98. Academic Press Inc., London.

SNEATH, P. H. A. (1979) Basic program for a significance test for two clusters in Euclidean space as measured by their overlap *Computers & Geosciences* **5**:143-155.

SNEATH, P. H. A. (1980) Basic program for determining overlap between groups in an identification matrix of percent positive characters. *Computers & Geosciences* **6**:267-278.

SNEATH, P. H. A., STEVENS, M. AND SACKIN, M. J. (1981) Numerical taxonomy of *Pseudomonas* based on published records of substrate utilization. *Antonie Van Leeuwenhoek Journal of Microbiology* **47**:423-448.

SOKAL, R. R. & SNEATH, P. H. A. (1963) *Principles of numerical taxonomy.* W. H. Freeman and Company, San Francisco.

SOKAL, R. R. & ROHLF, F. J. (1962) The comparison of dendrograms by objective methods. *Taxon* 11:33-40.

SOKAL, R. R. & MICHENER, C. D. (1958) A statistical method for evaluating systematic relationships. *Univ., Kansas Sci.* Bull. 38:1409-1438.

SORHAUG, T. & STEPANIAK, L. (1997) Psychrotrophs and their enzymes in milk and dairy products: Quality aspects. *Trends In Food Science & Technology* 8(2):35-41.

STACKEBRANDT, E. R., MURRAY, G. E. & TRUPER, H. G. (1988) *Proteobacteria classis nov.*, a name for the phylogenetic taxon that includes the purple bacteria and their relatives" *Int. J. Syst. Bacteriol.* 38:321-325.

STALEY, J. T. & KRIEG, N. J. (1984) Classification of prokaryotic organism an overview pp 1-3. In: *Bergey's manual of systematic bacteriology*, Volume I. N. R. Krieg and J. G. Holt (Eds). The Williams & Wilkins Co., Baltimore.

STANIER, R. Y., PALLERONI, N. J. & DOUDOROFF, M. (1966) The aerobic pseudomonads: a taxonomic study. *Journal of General Microbiology* 43:159-313.

STEAD, D. E. (1992) Grouping of plant-pathogenic and some other *Pseudomonas* spp by using cellular fatty-acid profiles. *International Journal of Systematic Bacteriology* 42(2):281-295.

STEPHENSON, M. (1939) *Bacterial metabolism.* Longmans, Green, London.

STRINGER, S. C., CHAFFEY, B. J., DODD, C. E. R., MORGAN, M. R. A. & WAITES, W. M. (1995a). Specific antibody-mediated detection of *Brochothrix thermosphacta in situ* in fresh British sausage. *Journal of Applied Bacteriology* 78:335-340.

STRINGER, S. C., DODD, C. E. R., MORGAN, M. R. A. & WAITES, W. M. (1995b) Locating nisin-producing *Lactococcus lactis* in a fermented meat system. *Journal of Applied Bacteriology* 78:341-348.

STRINGER, S. C., DODD, C. E. R., MORGAN, M. R. A. & WAITES, W. M. (1995c) Detection of micro-organisms *in situ* in solid foods. *Trends in Food Science and Technology* 6, 370-374.

STULL, T. L., LIPUMA, J. J., EDLIND, T. D. (1988) A broad-spectrum probe for molecular epidemiology of bacterial ribosomal-RNA. *Journal of infectious diseases* 157(2):280-286.

TERNSTROM, A., LINDBERG, A. M. & MOLIN, G (1993) Classification of the spoilage flora of raw and pasteurized bovine-milk, with special reference to *Pseudomonas* and *Bacillus*. *Journal of Applied Bacteriology* 75(1):25-34.

THOMPSON, J. M. (1996) Identification and differentiation of *Bacillus* from bakery origins. Ph.D. thesis, University of Nottingham.

TUYENBURG MUYS, G. (1971) Microbial safety in emulsions *Process Biochemistry*. 6(6):25-28.

VANCANNEYT, M., TORCK, U., DEWETTINCK, D., VAEREWIJCK, M. & KERSTERS, K. (1996) Grouping of pseudomonads by SDS-PAGE of whole-cell proteins *System Appl. Microbiol.* 19:556-568.

VANDAMME, P., HEYNDRICKX, M., DEROOSE, I., LAMMENS. C., DE VOS, P. & KERSTERS, K (1977) Characterization of *Bordetella* strains and related bacteria by amplified ribosomal DNA restriction analysis and randomly and repetitive element-primed PCR. *International Journal of Systematic Bacteriology* 47(3):802-807.

VANDAMME, P., POT, B., GILLIS, M., DE VOS, P., KERSTERS, K. & SWINGS, J. (1996) Polyphasic taxonomy, a concensus app roach to bacterial systematics. *Microbiological reviews* pp 407-438.

VIALE, A. M., ARAKAKI, A. K., SONCINI, F. C. & FERREYRA.,R. G. (1994) Evolutionary relationships among eubacterial groups as inferred from GroEL (chaperonin) sequence comparisons. *Int. J. Syst. Bacteriol.* 44:527-533.

WAYNE, L. G., BRENNER. D. J., COLWELL,R. R., GRIMONT, P. A. D., KANDLER, P., KRICHEVSKY, M. I., MOORE, L. H., MOORE, W. E. C., MURRAY, R. G. E., STACKEBRANDT, E., STARR, M. P. & TRUPER, H. G. (1987) Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int. J. Syst. Bacteriol.* 37:463-464.

WIMPENNY, J. W. T. (1981) Spatial order in microbial ecosystems. *Biological Reviews* 56:295-342.

WINTER, G. & MILSTEIN, C. (1991) Man-made antibodies. *Nature* 349:293-299.

WOESE, C. R., BLANZ, P., HAHN, C. M. & TANNER, R. S. (1984) What isn't a pseudomonad?: the importance of nomenclature in bacterial identification *System. Appl. Microbiol.* 5:179-195.

WOLFSON, L. M. & SUMNER, S. S. (1993) Antibacterial activity of the lactoperoxidase system a review. *Journal of Food Protection* 56(10):887-892.

WYATT, G. M. (1992) with contributions from H. A. Lee and M. R. A. Morgan. *Immunoassays for food poisoning bacteria and bacterial toxins.* Chapman & Hall, London.

ZILLIG, W., KELNK, H., PALM, P., PUHLER, G., GROPP, G., GARRET, R. A. & LEFFERS. H. (1989) The phylogenetic relation of DNA-dependant RNA polymerases of archaebacteria, prokaryotes and eubacteria. *Can. J. Microbiol.* 35:73-80.

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